Vol. 86, No. 3 June, 1944

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

PARAMECIUM BURSARIA: LIFE HISTORY. I. IMMATURITY, MATURITY AND AGE[1]

H. S. JENNINGS

(University of California at Los Angeles)

Introduction

The discovery of mating types or sex types in the ciliate infusoria has made it possible to breed and cross these Protozoa as readily as higher organisms. This has made possible under favorable conditions a renewed study of the problems of youth, age and death, particularly in relation to conjugation. These problems form the subject of the investigation of which a first installment is presented here.

The unit for examination in such studies is the clone rather than the single cellular individual. We deal with youth, age and death of clones, not of single cells only. The clone consists of all individuals derived by vegetative fission from a single ex-conjugant. The age of the clone is properly reckoned from the time of separation of the two ex-conjugants of the ancestral pair.

A number of investigators, beginning with Maupas (1888, 1889) have reported that there is, following the separation of the conjugants, a period of immaturity, during which multiplication by fission occurs, but conjugation does not occur. This is followed, according to these reports, by a period of maturity during which conjugation may occur. The period of maturity is said to be followed by a period of decline or degeneration, often spoken of as age or senescence. According to Maupas (1888) conjugation during this period of decline results in the death of the conjugants. The decline itself, without conjugations, also ultimately results in death. G. N. Calkins was long the outstanding representative of this general conception of the life history of the ciliate, though he did not, I believe, report that conjugation during the period of decline results in death.

However, a number of investigators have shown that in some of the ciliate infusoria and in certain other Protozoa, a clone may, under favorable conditions, continue indefinitely to multiply by fission without decline, degeneration or other indications of senescence (Woodruff, Metalnikoff, Bělař, Hartmann, Beers, Dawson and others). A detailed review of these investigations, with references to the literature, is found in Jennings, 1929.

[1] This work was aided by grants from the Carnegie Corporation and from the Committee on Research in Problems of Sex, of the National Research Council.

However, after these demonstrations as before, it continues to be observed that clones of ciliates, cultivated for long periods in the laboratory undergo gradual decline and ultimate degeneration and extinction. The graph of vitality, as indicated by the rate of fission, shows a gradually descending course, as illustrated for many cases in Jennings, 1929. The experience of the present writer in recent long-continued culture of *Paramecium bursaria* has been of this type.

What is the nature of this gradual decline and degeneration, in the cases where it occurs? What is its relation to environmental conditions, to ageing, to conjugation, to other developmental or genetic processes? It is with these and related questions that the present investigation deals. A method of detecting and following the progressive decline of clones was discovered in the fact that the mortality after conjugation commonly increases with the age of the conjugating clones. This has made possible a detailed study of the problems.

The fact that clones of *Paramecium bursaria* show, beginning immediately after separation of the conjugants, successive periods of immaturity, adolescence, maturity, and decrepitude in age, has been mentioned in previous papers (Jennings, 1939; 1939a; 1941; 1941a; 1941b; 1942). The phenomena have now been subjected to extensive cultural and experimental analysis. In presenting the results, the different periods of life will be successively taken up. Clones now or recently in the laboratory have been cultured for many different periods, up to eight years. Some of these clones are derived from individuals collected in nature; others were produced by conjugation in the laboratory. These numerous clones furnish abundant favorable material for examination of the pertinent questions.

The investigation has been devoted mainly to the genetic or intrinsic factors involved, though later installments will deal to some extent with the action of environmental factors. The method of work has been largely that of inducing conjugations within or between known clones, particularly at different ages, and following the history of the clones produced at these conjugations. Other results of such matings, particularly as to the inheritance of sex types or mating types, have been presented in detail in two previous papers (Jennings, 1941; 1942); those results form a foundation for much of the work to be presented in the series here begun.

The conditions in *Paramecium bursaria* that are particularly favorable for this type of work may be briefly recapitulated. Any variety of the species is differentiated into several mating types or sex types. One variety has eight sex types; two others have four each; a fourth variety seemingly has but two. In any clone all the individuals are of the same sex type, save in the infrequent cases of differentiation of a clone into two sex types (see Jennings, 1941). Mature clones of a given sex type conjugate readily with any of the other sex types of that variety (when their individuals are mingled together) but not with clones of their own sex type, nor with clones of other varieties. The readiness to conjugate and the mortality after conjugation vary with the laboratory age and with certain other conditions, as will be set forth.

The investigation has been an extensive and long-continued one. The author has been able to devote his time and energy to it exclusively, so that it has been the hope and design to devote so much time and attention to each problem that arose as to clear it up fully. This hope has been realized, I believe, for certain aspects of

the work, but also many questions have been raised that will require further work. The investigation deals with all the various periods of the life history of clones, but the chief problem for study has been age and natural death. The present first installment deals mainly, however, with the periods of immaturity and maturity, and with a general survey of the phenomena of ageing and death. Later installments will present detailed experimental investigations of ageing and death and of their relations to conjugation.

Culture Methods

The organisms were cultivated in the lettuce infusion impregnated with *Flavobacterium brunneum* (see Jennings, 1939; 1942). In the earlier years the alga *Stichococcus bacillaris* was added to the infusion (Jennings, 1939). In later years the alga was not used, as the organisms flourish equally well without it. Also for certain later years the infusion employed was diluted to one half the concentration described in 1939, but in the long run this does not work as well as the more concentrated infusion. For months the organisms flourish as well as they do in the concentrated infusion, but decline begins earlier in the dilute infusion. Therefore from 1943 the more concentrated infusion was used, as in the earlier years.

If the infusorians are under culture in a small vessel, such as a Syracuse dish, or in the deep depressions of thick culture slides, they continue to multiply vigorously if transferred to new culture fluid about once a week. If the cultures are left unchanged for considerably longer periods, some of them continue to flourish, but in most of them the animals cease to multiply after a time, and they become thin, at the same time decreasing in number. Why some old cultures continue to flourish, while most do not, is obscure. The infusoria may live in a depressed condition in the old cultures for long periods, reviving and becoming vigorous, with resumption of multiplication, when transferred to fresh infusion. There are indications that this environmentally-induced depressed condition is due in some considerable measure to the unfavorably altered condition of the infusion, possibly to deleterious products of metabolism from either themselves or the bacteria on which they feed, rather than to mere lack of food. In any case it is important to distinguish such temporary and purely environmental depressions from the depressed conditions of the clone that is largely independent of present environment, as described later. Whether however there is a connection between the two—whether long-continued extrinsic environmental depression may tend in time to induce intrinsic or genetic depression—may remain for the present an open question.

Clones differ greatly in their resistance to unfavorable cultural conditions. Some die out quickly under conditions in which others continue to exist and from which they recover when the conditions improve. Particular clones, indeed, differ at different periods of their lives in their resistance to unfavorable conditions: a matter which forms in large measure the subject of the present investigation.

The clones that have been cultivated for long periods in our laboratory have as a rule been subjected at intervals, particularly in the earlier years, to periods of environmental depression. This appears to be of importance in connection with the long periods of immaturity shown by some of the clones, as will be set forth in a later section.

Special methods will be described in connection with the phenomena in relation to which they are employed.

Immaturity: Its Dependence on Cultural Conditions

For a period of time after conjugation the descendants of the ex-conjugants neither form clots nor conjugate, even though brought under favorable conditions into intimate contact with individuals of different sex type, belonging to the same variety. This period of sexual immaturity varies greatly in length in different clones, even among those produced by conjugation of the same two parental clones. Immature periods varying in length from a minimum of twelve days up to some years have been observed (see Jennings, 1939, pp. 214–215). There is among our clones one ("403*a*1") that was produced October 20, 1937 (by self fertilization of a clone of Variety I); it still does not conjugate (July 1943) though more than five years old. This clone that has never become mature has a well developed micronucleus (T. T. Chen, personal communication); it has now (1943) passed into a period of depression.

The great variation in length of the period of immaturity, taken in connection with the fact that many or most of the clone cultures were subjected at times to the depressing conditions mentioned above, suggested that possibly the age at which sexual maturity is reached depends to some extent on the cultural conditions. This was tested in the following way:

By conjugation between two clones a large number of pairs was obtained. From these pairs were derived many ex-conjugant clones (two from each pair). Each of these ex-conjugant clones was divided, after it had multiplied to some extent, into two parts. One part was kept in vigorous multiplication by changing and renewing frequently the culture fluid, and by keeping the number of individuals small; this is known as the rapid portion (R). The other part of each clone was subjected to unfavorable conditions, which interrupted or made very slow the multiplication by fission; this is known as the slow portion (S). This was done by allowing the cultures to become crowded, and the culture medium to become old. There were thus genetically identical sets of R and S clones, with many clones in each set. After a period of this diverse culture, the two sets of clones were restored to the same favorable cultural conditions. Here they were allowed to multiply, and at intervals the clones of both sets were tested in the usual way to determine if they were sexually mature. That is, the two divisions (R and S) of each clone were tested each with the four mature sex types of the variety (Variety I). Clones that were mature reacted by forming clots and pairs with three of the four sex types; those that were immature did not form clots or pairs.

Two extensive experimental cultures of this type were carried through; both showed that the cultural conditions in which a clone has lived do indeed affect the age at which it becomes mature. Each experiment lasts for many months and requires several or many successive tests of each clone, at intervals of weeks or months.

The first experiment was begun May 9, 1941, at which time 24 pairs were obtained from the conjugation of the two clones 39 and 44, respectively of the sex types D and B of Variety 1. (Clone 39 was collected December 23, 1939 at Monterey, California; clone 44 March 6, 1941, at Westwood Village, Los Angeles,

California.) After separation the two ex-conjugants of each pair were isolated, so that there were 48 ex-conjugant clones. Each was allowed to multiply separately in fresh culture medium until May 20 when a considerable number of individuals was present in each of 46 of the clones (two of the 48 ex-conjugant clones were non-viable).

Now each of the 46 ex-conjugant clones was separated into two parts: a "rapid" part, designated R and a "slow" part, S. There were now therefore 92 separate cultures, two for each of the 46 clones. Each "rapid" culture, R, was begun with but ten individuals, while its corresponding "slow" culture, S, included at the beginning a large number of individuals (the remainder of the clone). The rapid cultures were kept in vigorous multiplication; every second day four individuals of each were employed to carry on the culture, and these were transferred to abundant fresh culture medium, the rest of the culture being discarded. In the slow cultures of the 46 clones, on the other hand all the individuals were left in each case on the depression slide and were there allowed to multiply till they became so numerous that reproduction nearly or quite ceased and the individuals became thin. As the culture fluid evaporated, fresh weak fluid was added, at intervals of several days, but there was in these slow cultures no transfer to fresh slides or infusion. Thus the slow cultures were kept for a long period in a depressed condition. Their individuals remained normal in appearance, but were slender and did not become numerous.

At later periods both the rapid and the slow were placed in fresh fluid in Syracuse dishes, and were allowed to multiply there till they became sufficiently numerous to be tested for maturity. At the time of testing, therefore, the two sets were living under the same conditions and were both multiplying well. Does the earlier different history of the two make a difference in the age at which maturity is reached?

The first test was made June 6 to June 23, 1941, when the clones were about one month old. The first 12 clones were tested from each of the two sets of cultures, the tests being carried out in the usual way. None were yet mature; no clots or pairs were produced.

The next test was August 7 to 16, 1941. Five of the slow cultures had succumbed and died out; the corresponding rapid cultures of the same clones were discarded. This left 41 clones, each represented by a rapid and a slow culture. All those in the rapid cultures except four had now become sexually mature, as shown by their reaction (production of clots and pairs) with the test clones. But none of the slow cultures reacted; none was mature.

Thus the difference in cultural conditions had produced a great difference in the age at which the organisms became sexually mature. At the age of 3 months those parts of the 41 clones that had been kept vigorously multiplying were all mature except four, while their genetic duplicates that had been subjected to depressing conditions were all still sexually immature.

The four clones of the rapid set that did not react in the August tests became mature in October. At the age of 5 months all the 41 clones in their rapid cultures were mature.

From the time of the August tests the slow cultures were tested at intervals of about one month. Some additional cultures died out, so that 33 clones were tested

in the slow cultures in October; none were yet mature. In the latter half of November 1941, the slow cultures of 30 clones were tested, one had now become mature. No additional culture of the slow set had become mature at tests in December 1941, January 1942, February 1942, or March 1942.

In July 1942, 14 months after conjugation, the still surviving cultures of the slow set were again tested. Only eight clones were now represented in the eight living cultures of the slow set. Of these, four were now fully mature, while two others were becoming mature, since they reacted with two of the four sex types of the variety. The other two cultures were still immature.

Thus while clones kept in vigorous multiplication all become mature at the age of 3 to 5 months, the same clones subjected to depressing conditions became mature only at 10 to 14 months or later.

It appeared desirable to repeat this experiment. The second experiment began October 22, 1941, at which time were isolated 54 pairs, produced by the conjugation of two clones derived from two different pairs of the "rapid" set of the experiment just described. On October 27 each of the 108 ex-conjugant clones from these 54 pairs was divided into two parts, a "rapid" lot (R) and a "slow" lot (S). The rapid set was cultured, as in the previous experiment, in such a way as to keep the individuals multiplying rapidly. The slow set (duplicate of the rapid) was subjected, as before, to crowding and starvation. There were thus 108 clones in each set.

The cultures of the "slow" set were subjected to depressing conditions, in this case, for but 18 days, till November 14, 1941. On this date all the surviving clones, R and S, were placed in Syracuse dishes, and thenceforth all were cultivated in the same way, in the usual dilute lettuce infusion. The point to be determined is whether the cultivation of one of the duplicate sets under depressing conditions for 18 days, while the other set was vigorously reproducing, has made a difference in the age at which sexual maturity comes on.

In this second generation experiment the mortality of the clones was much greater than in the previous generation. Of the total 216 ex-conjugant cultures (sum of the R and the S), only 91 survived to be tested. These included the same clone in both sets in 37 cases (37 R and 37 S that were genetically identical). In addition there were 5 clones that were represented in the R set but not in the S set, while 12 other clones were represented in the S set, but not in the R set. That is, 42 clones were represented in the R set, 49 in the S set, and 37 of those in each set were the same clones.

In the 37 clones that were common to the two sets, the rapid culture became mature in every case before the slow culture of the same clone. In 22 of the 37 cases only the rapid culture became mature within the 8 months during which the experiment was continued, the same clones in the slow cultures remaining immature to the end. In the other 15 clones of the 37 that were represented in both sets of cultures, both cultures became mature, the rapid culture in every case a month or more earlier than the same clone in the slow set.

Up to the end of February 1942, when the clones were a little more than 4 months old, 42 clones of the R set had been tested, and 36 were found to be mature; that is, in the rapid cultures 85.7 per cent of the clones were mature at the end of 4 months. At the same date 46 of the 49 clones in the slow set had been tested, of

which 14 were found to be mature, so that but 30.4 per cent of the clones in the slow cultures were mature at the age of 4 months. At the end of June, when the clones were 8 months old, 40 clones were mature of the 42 in the rapid cultures (95.2 per cent) while at the same time but 20 of the 49 clones in the slow cultures were mature (40.8 per cent).

Thus these two long-continued experiments give the same result. Clone cultures that are kept vigorously multiplying, without starvation or crowding, or staleness of the culture medium, become mature much earlier than the same clones in cultures that have been subjected for a considerable period to the depressing effects of the conditions just named. Those kept vigorously multiplying usually become mature in 3 to 4 months, though there is much variation among them, and in some the immature period is much shorter. Those that have been subjected to depressing influences such as starvation, crowding and staleness do not become mature till they are 10 to 14 months of age, or older. In both the experiments described above a number of the clones thus subjected to depressing conditions did not become mature during the time the experiments continued.

In these phenomena we see temporary differential action of diverse environments producing in the stocks differences that continue for months of vegetative reproduction after the differential environmental action has ceased. In the second experiment described above the effectively diverse environmental conditions lasted for but 18 days, but the two sets thus diversely acted on retained the induced diversity for at least 7 months. The induced diversity is transmitted in vegetative reproduction for many successive generations.

It has been suggested verbally to me that possibly the time of becoming mature depends on the number of vegetative generations that have passed since conjugation. Depressing conditions decrease the number of vegetative generations passed through in a given period of time, and would therefore, if the above suggestion is correct, lengthen the time to maturity. But in view of the great difference in the time of becoming mature induced by a relatively short period of depressing conditions, the above suggestion appears not probable, and it is more likely that the action of depressing conditions is more direct, producing physiological changes that delay the attainment of maturity more or less independently of the number of vegetative generations that have passed. The matter is one that is worthy of precise and detailed experimental study, as indeed is the entire phenomenon of the relation of the attainment of maturity to environmental conditions.

The facts shown by the experiments are of practical importance for the investigator. For many purposes it is necessary to cultivate the ex-conjugant clones until they are mature, in order that needed crosses may be made. The time required for this is greatly shortened if the clones are kept continuously in vigorous multiplication. It appears probable that the frequent very long periods of immaturity in my own work mentioned in earlier papers, were due to the fact that in their culture the clones were at times subjected to depressing conditions.

There is an important additional relation observed on comparing the rapid and slow subdivisions of the clones. Though clones produced by different pairs are frequently of different sex types, the two subdivisions of any single ex-conjugant clone are always finally of the same sex type, in spite of the fact that they have been cultured differently and have become mature at widely different periods. In the

two experimental cultures of which account is given above, there were 21 ex-conjugant clones in which the sex-type was determined for both the rapid or early maturing, and the slow or late maturing subdivisions. Of the 21 ex-conjugant clones 13 were of sex type A, five were B and three were C. In all cases both subdivisions of any clone were of the same sex type. This agrees with all other evidence in showing that the sex type is determined genetically and is not ordinarily altered by changed environmental conditions.

Transitional Period from Immaturity to Maturity

In the life of most clones there is a period of transition, during which the ex-conjugant clone reacts sexually only in sporadic individuals, and in some cases with only one or two of the sex-types of the variety to which it belongs. This transitional period may last for weeks. Details, with many examples of the sporadic sex reactions, have been given in the third report on these investigations (1942, pages 196–199). Rarely the transitional period is short, or possibly entirely lacking, the clone suddenly acquiring the typical strong reaction in all (or most) of its members, with the usual clot formation and resultant numerous pairs.

Maturity

The sexually mature period is characterized in typical cases by the fact that when clones of different sex type are mingled, there occurs the strongly marked clotting followed by formation of numerous pairs (Jennings, 1939).

It is notable, however, that the strength of the tendency to clot varies greatly in different clones. Some clones when mixed form at once large clots, like those photographed in the 1939 paper. Others form but small clots, containing only a few individuals (three or four or less). In some clones only a few individuals take part in the clotting, while in others all are active in the sexual reaction. Some clones do not react at once when mixed, but do react later. Some do not react on the day the mixture is made, but react (strongly or weakly) the next day.

These differences in the tendency to clot resemble those described by Moewus in certain flagellates, which Moewus has correlated with different genetic constitutions. (For summaries of this work of Moewus, the paper of Sonneborn, 1941, may be consulted.) These phenomena are worthy of detailed study in the ciliates.

The period of sexual maturity lasts for several years, and is followed by a period of decline which forms the chief subject of the present investigation.

A remarkable phenomenon is to be observed at times in mature clones. When two clones of different sex type (but of the same variety) are mingled, strongly marked clotting usually occurs, the individuals coming into intimate contact; but in some cases no conjugated pairs are finally produced. The clotting occurs during the day; toward evening the clots break up, not into pairs, as in the normal case, but into separate single individuals. In the normal case, after clotting has occurred, many united pairs are found to be present in the mixture the next morning, before the new clotting of the second day has begun. These pairs remain in union for 36 hours or more. But in exceptional cases no pairs are present the next morning. On the second day clotting may occur again, but as before no lasting pairs are

formed. Thus the first stages of the mating reaction occur, but the final stages do not; conjugation is not completed.

A similar phenomenon has been described by Sonneborn (1942) in certain clones of *Paramecium aurelia*. In *Paramecium bursaria* such clotting without formation of pairs may at times characterize many clones of a collection. Such cases are the following: In February 1943 collections were made from ponds in the Botanical Garden of the University of California at Los Angeles; also from ponds in the Municipal Park at Beverly Hills. A considerable number of these clones showed clotting without formation of lasting pairs, presenting opportunity for a study of the phenomena.

From the two collections 46 clones were isolated and cultivated. These included representatives of all the four sex-types of Variety I. Three clones were of sex type A, nine of type B, 24 of type C, and ten of type D. Representatives of each clone were tested with all clones of the three types to which the clone under test did not belong. In all cases clots were formed, but a considerable number of the mixtures did not form lasting pairs. All clones of sex types A and D (13 clones in all) formed clots and pairs in the normal way, when mixed with clones that were capable of forming pairs. Of the nine clones of the B type, four were normal, forming clots and pairs in the usual way, while five ordinarily did not form pairs, even when mixed with clones that were themselves thoroughly normal. All these form clots as usual, though no pairs. Of the 24 clones of C type, seven formed clots and pairs in the usual way, while 17 formed clots but did not form pairs. Thus of the 46 clones 24 formed clots succeeded by pairs in the usual way, while 22 clones formed clots but did not form pairs (save in isolated instances; see next paragraph).

A peculiar feature of the phenomena is that certain of the clones that as a rule do not form pairs may in certain instances form one or two pairs. This occurred in mixtures in which in normal reactions there would be found as many as a hundred or more pairs. Such isolated pairs were observed in five of the clones that commonly formed no pairs. Three of them were of type C, while two were of type B. In each of four of these five clones but a single lasting pair was observed, though there were for each clone several mixtures in each of which many pairs would in the normal case be formed. In the fifth clone ("BH130") no pairs were formed in mixtures with any of the 37 clones of different sex types that belong to these collections; but three pairs were formed when individuals of this clone were mixed with certain "tester" clones (of types A and D), that had been selected because of their strong sexual reactions.

To determine whether a micronucleus is present in the clones, particularly in those that do not form pairs, a cytological examination was made by Dr. T. T. Chen. This examination covered 41 of the 46 clones in the collection mentioned above; it included 19 of the clones that do not ordinarily form pairs, and 22 of those that do. A micronucleus was found to be present in all the clones that regularly form pairs; also in 15 of the 19 clones that clot but do not form pairs. Four clones were without micronuclei, and all of these belonged to the group that clot but do not ordinarily form pairs. Two of them had produced an isolated pair or two in certain mixtures.

Absence of a micronucleus thus usually, though not always, prevents pair forma-

tion, though it does not prevent clotting. But other conditions may prevent formation of pairs, since 15 clones that had micronuclei did not form pairs.

The absence of a micronucleus does not prevent the manifestation of the sex type. Of the four clones without micronuclei, three belonged clearly to sex type B, while the fourth was a well defined type C.

In the other 37 clones the micronuclei varied considerably in form, size and stainability. But no special characteristics appeared peculiar to the 15 clones that did not form lasting pairs.

The cytological changes in this clotting without conjugation, or "formation of temporary pairs" have been examined by Dr. T. T. Chen, who will report on them in a separate paper.

Age, Mortality, and the Consequences of Conjugation

We have in the laboratory many clones of the different varieties of *Paramecium bursaria*. In January 1943 there was under culture a reserve stock of 264 clones, in addition to many recent ex-conjugant clones that were under immediate study. The clones are of many different ages. Some have been under cultivation for about eight years (April 1943). Others vary from six years to but a few months or days of laboratory cultivation. Many of the clones were collected in nature, from diverse parts of the United States or abroad. Others were produced in the laboratory by the conjugation of pre-existing clones.

Many of these clones that at first flourished vigorously have since declined in vigor, with alteration of many of their life phenomena. Some have died out, in spite of the greatest care in cultivation. Many of the old clones at conjugation produce pairs of which the majority die. These observations formed the starting point of the present investigation.

Symptoms of Degeneration as Clones Become Old

Our oldest clones, Nos. 1 (or "m") and 2 (or "l") were collected April 18, 1935, at Alexandria, Virginia. At the time they came into my hands, in June 1937, they had been maintained for two years in the laboratory by Dr. T. T. Chen. What their age may have been at the time of collection there is of course no way of knowing, so that the total age of each is unknown. The two have shown in the later years depression or degeneration manifested in a number of different ways. Their history is instructive.

When they came into my hands in June 1937 Clones 1 and 2 were vigorous in vegetative reproduction, but showed a high mortality at the time of conjugation. The two were of different sex type (No. 1 of type C, No. 2 of type A), and were bred together in June and July 1937. From their mating 142 pairs were obtained, yielding, after separation of the conjugants, 284 ex-conjugants. Of these, descendants of but 18 ex-conjugants survived and formed clone cultures; that is, but 6.3 per cent of the ex-conjugant clones survived.

The clone No. 1 was divided into many cultures which were cultivated separately. In January 1938 certain of these cultures yielded pairs by self differentiation and self fertilization. There were 118 of these pairs from the selfing of clone Number 1, yielding 236 ex-conjugants. Again the mortality was excessively high,

only six of the 236 surviving and forming ex-conjugant clones. Thus but 2.5 per cent of the ex-conjugants from the selfed clone No. 1 survived.

Not all of the cultures No. 1 underwent self differentiation and self fertilization. Those that did so contained in consequence individuals of the two mating types C and B, which conjugated, yielding pairs as just set forth. But several cultures remained of the pure original type C. In order to insure that the cultures should contain only this type, new cultures were started from single individuals, of the mating type C.

In February 1940 it became apparent that the cultures of clone No. 1 were less flourishing than those of other clones. By October of that year all cultures of this clone had become scanty, and when new cultures were seeded with a number of individuals of the clone there was little multiplication. The contrast with other clones in this respect was striking. Individuals of abnormal form made their appearance in the cultures of clone No. 1. A number of the cultures died out.

From October 17 to October 28, 1940, a period of 12 days, comparative isolation cultures were carried on of this clone No. 1 and of three other clones (see Table I). These were for the purpose of comparing the rate of fission and the

TABLE I

Paramecium bursaria; rates of multiplication and frequency of deaths in certain clones, in comparison with Clone 1, for the period October 17 to October 28, 1940.

Each clone consists of 24 parallel lines cultivated for 12 days. The number of fissions in the 24 different lines during the 12 days is summarized; also the total number of deaths of lines in each clone of 24 lines.

Clone 1 (Laboratory age 66 months). Number of fissions in the different lines varies from 0 to 7. Number of deaths, 14.

Clone 2 (Laboratory age 66 months). Number of fissions, 17 to 23. Number of deaths, 4.

Clone 6 (Age 40 months). Number of fissions, 20 to 27. Number of deaths, 2.

Clone 36 (Laboratory age 11 months). Number of fissions, 27 to 30. Number of deaths, 1.

frequency of deaths in clone 1 with those of the three other clones. Twenty-four lines of each clone were cultured on depression slides. Each of the 24 lines of each of the four clones was begun as a single individual in one of the depressions of the slides. Each was allowed to multiply for 24 hours; then the number present was recorded and a single individual from each depression was transferred to a new slide and fresh fluid, and allowed to multiply for 24 hours as before. This was repeated for each of the 96 lines throughout 12 days. At the end of the 12-day period there were records for the 24 lines of each of the four clones. In Table I are given the number of fissions in the lowest and highest line of each clone; in other words, the range of variation in fissions for the 24 lines of the clone. Table I also gives the number of deaths in the 24 lines of each clone during the 12-day period. This number of deaths was obtained as follows: After the daily transfer of a single individual to a new slide, sometimes this individual died, ending the line. The line was then continued by substituting an individual from one of the other lines of that clone. The "number of deaths" in Table I shows how many times this occurred in the 24 lines of each clone.

The clones compared with clone 1 in Table I are the following:

Clone 2 was collected at the same time and place as clone 1, and hence was of

the same laboratory age. It was not vegetatively depressed at the time of the cultures of Table I.

Clone 6 was derived from a pair resulting from the conjugation of clone 1 with clone 2, June 18, 1937. It is therefore younger than its parent clones 1 and 2 by somewhat more than 2 years.

Clone 36 was collected at Los Angeles, November 18, 1939.

Thus some of the lines of clone 1 did not divide at all during 12 days, and the most vigorous line divided but seven times. In contrast, the other three clones, living under exactly the same conditions, multiplied at the rate of one, two, or more fissions daily, in each line. The clone 1 shows many deaths and hardly multiplies at all.

During November 1940 it became increasingly difficult to keep the cultures of clone 1 alive. There were many such cultures, some on slides, some in Syracuse dishes. Some were cultivated in the dilute lettuce infusion, some in the more concentrated, some with algae, some without. Under all these conditions other clones multiplied vigorously. But one after another the cultures of clone 1 died out, until on January 26, 1941 the last culture died and the clone 1 became extinct.

A similar downward course has been followed by certain other clones. Some have become completely extinct; others still exist as weak scanty cultures that are kept alive only with difficulty. All this has occurred under conditions in which other clones flourish. Some examples may be mentioned.

The clone "McD3" was collected near Baltimore, Maryland, February 7, 1938. It was long one of the most vigorous of the clones in the laboratory, and was employed as the chief tester of sex type M, Variety II. But it became weak and degenerate, and for many months in 1942 and 1943 it was kept alive only with difficulty. It finally died out, in all its cultures, in March 1943.

A considerable number of clones of Variety III were obtained, some from North Carolina, February 25 and March 20, 1938; some from Provincetown, Massachusetts, July 28, 1938. By crosses among the clones from North Carolina many additional clones were obtained. Some 24 clones were kept under culture as a reserve stock. Now after more than 5 years almost all of these stock clones have died out. Before final death they were for many months in bad condition, multiplying little and showing hardly any tendency to sexual reaction. The few clones that remain are in bad condition and will doubtless soon die. As Variety III is not known to occur on the Pacific slope and has rarely been collected elsewhere, the loss is a serious one, leaving the laboratory without testers for Variety III.

A peculiar variation in these histories is shown by the clone known as S of Variety II. It was collected in the spring of 1937. For years it flourished and was employed as a tester of the sex type J. In 1940 to 1943 it became very weak and many of its cultures died out. All of those which were kept rapidly multiplying died, but a certain old quiescent culture, in which the animals were thin and multiplied little or not at all, has remained alive. Attempts to bring its members to rapid multiplication by additions of fresh culture fluid results in their immediate death.

Many other cases of decline or degeneration of clones after they had long been under culture have been followed in this laboratory. A large number of stock cultures were long kept on hand. These were transferred weekly to new cultures.

For a long time they flourished, now more than half of them have died out (June 1943).

Age of Clones in Relation to the Readiness to Conjugate and to Fertility and Mortality in Conjugation

Observations on decline in old clones such as set forth above left in many cases the further impression that old clones may conjugate but that the mortality after such conjugations is greater than usual, many of the ex-conjugants dying without the production of long lived clones. Maupas in his great papers of 1888 and 1889 was left with a similar impression. He gives many instances of high mortality at conjugation in Stylonychia, in Onychodromus, in Leucophrys, in Didinium, in Spirostomum. In some of these cases the stocks were known to be old; in others this was uncertain. In some cases Maupas attributed the high mortality to the supposed or known fact that the conjugating animals were close relatives. In view of these impressions and the accounts given by Maupas, an extensive investigation was undertaken of the relation both of age and of inbreeding to mortality at conjugation. The results of this work will be presented in full in later papers.

Since the time of Maupas the only work bearing on this precise matter appears to be that on mortality at endomixis in *Paramecium aurelia*, carried out mainly by Sonneborn or under his inspiration, and published by Jennings and Sonneborn, 1936, by Gelber, 1938, and Pierson, 1938. At the time the work was done it was not known that "endomixis" is a form of conjugation, or closely related to it, as "autogamy." But Diller (1936) showed for *Paramecium aurelia* that in "endomixis" two nuclei unite, both produced of course by the single individual, so that the process is a close form of self-fertilization; and this was confirmed genetically by Sonneborn (1939). (Whether in all cases endomixis includes such autogamy is extensively discussed by Woodruff, 1941.) Jennings and Sonneborn (1936) showed that lines which omit endomixis die, and that many die at the occurrence of endomixis; also that "It appears that the longer endomixis is omitted the greater the proportion of individuals that die when they undergo it" (p. 419). This relation to the time elapsed since the foregoing endomixis was examined statistically by Gelber (1938) and Pierson (1938). They showed that the older the lines, reckoned from the last endomictic period, the greater the proportion of deaths at endomixis. Comparison of their results with those on conjugation in *Paramecium bursaria* will be presented in later installments of the present investigation.

Readiness to conjugate in old clones: In clone No. 1, above described, it was found that the individuals were ready to conjugate long after the time when the clone had become weak; it continued till very shortly before the death of the clone. In mixtures of clone No. 1 (sex type C) with clones of types A, B and D, clotting occurred January 5, 1940; though all cultures of clone No. 1 died out January 26 of that year. It was notable, however, that though typical clotting occurred, in but few cases were pairs formed and conjugation completed. From the abundant clotting of January 5 but four firmly united pairs persisted till the next morning. Three of them separated that morning, indicating that conjugation had not been consummated. The fourth separated at the normal time, later, but of the two ex-conjugants one died without fission, the other divided but once, then both its descendants died.

Other old clones formed clots and later pairs, when mixed with clones of other sex types. The fate of these pairs is to be taken up later.

Thus old clones of Variety I undergo clotting and pairing with other sex types up to a short time before their death from age; a result that agrees with the observations of Maupas on other species.

Age in relation to mortality at conjugation: In most epidemics of conjugation there is a certain amount of mortality among the ex-conjugants or their immediate descendants. When the clones that conjugate are old the percentage of mortality is high, as seen in certain instances cited above.

The relation of mortality at conjugation to age of the conjugating clones presents opportunity for experimental study of the nature and progress of ageing. It has therefore been subjected to extensive and intensive investigation. The data on this and the conclusions to be drawn are to be presented in papers soon to appear.

Summary

The life history of clones of *Paramecium bursaria* shows successive periods: (1) a period of sexual immaturity, during which sexual reactions and conjugation do not occur: (2) a transitional period during which weak sexual reactions occur in a few individuals: (3) a period of maturity, in which sexual reactions are strongly marked and the individuals conjugate readily: (4) a period of decline, ending in many (or all?) cases in death.

The length of the period of immaturity and the time of attainment of maturity depend on the cultural conditions. If the animals are kept rapidly multiplying, under the best of nutritive conditions, maturity comes on early; if they are subjected to periods of starvation or other depressing conditions, maturity comes on much later or not at all.

Ex-conjugant clones that are kept vigorously multiplying become mature in most cases at the age of three to five months, though cases have been observed of much earlier maturity, the earliest observed age of maturity being 12 days.

Ex-conjugant clones subjected for some time to depressing conditions become mature (even after restoration to favorable conditions) only at the age of 10 to 14 months. Certain clones have lived for years without becoming mature.

If single ex-conjugant clones are divided into two cultures, one subjected to conditions favorable to rapid multiplication, the other to unfavorable conditions, the two parts show these same differences. The part kept under favorable conditions matures months before the other part.

Subjection to depressing conditions for but short periods (18 days) delays maturity for months.

Thus temporary differential action of diverse environments produces in clones differences which persist through months of vegetative reproduction.

In the period of maturity, clotting and conjugation *en masse* are commonly produced when cultures of clones of different sex type are mixed. But in certain clones clotting occurs without the completion of conjugation; dense clots occur, but no pairs are formed.

The period of maturity lasts for several years. It is followed by a period of decline. In this period fission becomes slower; abnormalities appear; many individuals die, so that the cultures become scanty and finally die out completely.

Clones have been cultivated in the laboratory five to eight years, finally showing degeneration and death.

During the period of decline conjugation may occur up to near the very end. But conjugation of aged stocks results in the death of most or all of the ex-conjugants.

The relation of age to mortality at conjugation presents many features of interest, and gives opportunity for study of the nature and progress of ageing. This matter is to be presented in later contributions.

LITERATURE CITED

DILLER, W. F., 1936. Nuclear reorganization processes in Paramecium aurelia, with descriptions of autogamy and 'hemixis.' *Jour. Morph.*, **59**: 11–66.

GELBER, J., 1938. The effect of shorter than normal interendomictic intervals on mortality after endomixis in Paramecium aurelia. *Biol. Bull.*, **74**: 244–246.

JENNINGS, H. S., 1929. Genetics of the Protozoa. *Bibliographia Genetica*, **V**, pp. 105–330.

JENNINGS, H. S., 1939. Genetics of Paramecium bursaria. I. Mating types and groups, their interrelations and distribution; mating behavior and self-sterility. *Genetics*, **24**: 202–233.

JENNINGS, H. S., 1939a. Paramecium bursaria; mating types and groups, mating behavior, self-sterility; their development and inheritance. *Amer. Nat.*, **73**: 414–431.

JENNINGS, H. S., 1941. Genetics of Paramecium bursaria. II. Self-differentiation and self fertilization of clones. *Proc. Amer. Phil. Soc.*, **85**: 25–48.

JENNINGS, H. S., 1941a. The beginnings of social behavior in unicellular organisms. *Science*, **92**: 539–546. (Also separate; University of Pennsylvania Bicentennial Conference, 17 pp.)

JENNINGS, H. S., 1941b. The transition from the individual to the social level. *Science*, **94**: 447–453.

JENNINGS, H. S., 1942. Genetics of Paramecium bursaria. III. Inheritance of mating type, in crosses and in clonal self fertilizations. *Genetics*, **27**: 193–211.

JENNINGS, H. S., AND T. M. SONNEBORN, 1936. Relation of endomixis to vitality in Paramecium aurelia. *Comptes rendus du XIIe Congrès International de zoologie*, Lisbonne, 1935: 416–420.

MAUPAS, E., 1888. Recherches expérimentales sur la multiplication des infusoires ciliés. *Arch. d. Zool. Exp. et Gén.*, (2), **6**: 166–277.

MAUPAS, E., 1889. La rajeunissement karyogamique chez les ciliés. *Arch. d. Zool. Exp. et Gén.*, (2), **7**: 149–517.

PIERSON, BERNICE F., 1938. The relation of mortality after endomixis to the prior interendomictic interval in Paramecium aurelia. *Biol. Bull.*, **74**: 235–243.

SONNEBORN, T. M., 1939. Genetic evidence of autogamy in Paramecium aurelia. *Anat. Rec.*, **75**: 85.

SONNEBORN, T. M., 1941. Sexuality in unicellular organisms, Chapter 14, pp. 666–709, in *Protozoa in biological research* (Calkins and Summers, editors).

SONNEBORN, T. M., 1942. Evidence for two distinct mechanisms in the mating reaction of Paramecium aurelia. *Anat. Rec.*, **84**: 92–93.

WOODRUFF, L. L., 1941. Endomixis. Chapter 13, pp. 646–665 in *Protozoa in biological research* (Calkins and Summers, editors).

THE EFFECT OF FOOD CONTENT AND TEMPERATURE ON RESPIRATION IN PELOMYXA CAROLINENSIS WILSON[1]

D. M. PACE AND W. H. BELDA

(Department of Physiology and Pharmacology, College of Pharmacy, University of Nebraska, Lincoln; Department of Biology, Saint Francis Seminary, Milwaukee)

Introduction

Pelomyxa carolinensis Wilson (*Chaos chaos* Schaeffer) is an organism favorable for the study of cellular functions. It is one of the largest of the amoeboid forms, it can be cultured easily in the laboratory, and it is relatively simple in structure and physiological activity. Although it has been available from stock culture since 1937, apparently no studies have been made on the respiration of this organism; in fact, very few investigations have been made on respiration in any of the amoeboid forms.

Emerson (1929), using Barcroft-Warburg manometers, found that the oxygen consumption of *Amoeba proteus* at 20° C. amounts to 0.16 mm.^{3} per mm.^{3} of cell substance per hour. Howland and Bernstein (1931), using a modification of the capillary tube method devised by Kalmus (1928), found that the oxygen consumption of *Actinosphaerium eichhornii* at 20° C. is of the order of 1,100 mm.^{3} per hour per million organisms. They did not express the rate of oxygen consumption per unit volume of protoplasm.

The following investigations were carried out to ascertain the rate of respiration in *Pelomyxa carolinensis* under various conditions of nutrition and at different temperatures.

Material and Methods

The organisms used in these experiments were from a strain which has been cultured in the laboratory for several years in the manner described by Belda (1942). The pelomyxae were grown in Hahnert's (1932) solution, but prior to each experiment the organisms were kept for about a week in a culture medium buffered to maintain a hydrogen-ion concentration of pH 6.8. To keep this value constant, a high concentration of phosphates was required. Pelomyxae, put into experimental solutions containing only potassium and magnesium compounds, quickly disintegrated. However, the addition of calcium chloride served to counteract the toxic effects of the potassium ions, and the pelomyxae grew well in this medium. The formula which was finally adopted is given in Table I.

The pelomyxae were kept in glass finger bowls containing 150 ml. of buffered solution. Food was supplied for the organisms by adding portions of a centrifuged culture of *Paramecium caudatum* until there were about 600 to 700 paramecia per ml. Under these conditions the pelomyxae ingest from one to three paramecia

[1] These investigations were partially supported by a grant from the Penrose fund of the American Philosophical Society.

TABLE I

Buffered culture solution for Pelomyxa carolinensis

K_2HPO_4	80 mg.
KH_2PO_4	80 mg.
$CaCl_2$	104 mg.
$Mg_3(PO_4)_2 \cdot 4H_2O$	2 mg.
H_2O (redistilled)	1,000 ml.

within an hour, and continue feeding as long as any paramecia remain. Specimens of Pelomyxa were removed from the finger bowls at intervals for respiration studies.

The rate of oxygen consumption and carbon dioxide elimination was measured by means of a Barcroft-Warburg apparatus. The shaking rack held seven manometers and flasks, of which one was used as a thermo-barometer. Preliminary tests had shown that there was no measurable difference in the rate of oxygen consumption between pelomyxae tested in flasks which contained 100, 200, or 300 organisms. In the experiments as carried out, groups of either 100 or 200 specimens were used in each test.

To measure the rate of oxygen consumption, 0.4 ml. of 10 per cent KOH was put into the inset and 0.3 ml. of 3N HCl into the onset of each flask. To measure both the rate of oxygen consumption and of carbon dioxide elimination, the manometers were paired and 0.4 ml. of distilled water was put into the insets of half of the flasks and 0.4 ml. of 10 per cent KOH into those of the other half. The water bath of the apparatus was kept at the temperature selected for each experiment with a variation of not more than $\pm$ 0.05° C.

The manometers were mounted on a shaking mechanism which was operated at the rate of 124 complete cycles per minute through an amplitude of 3 cm. This amount of motion provided a sufficiently rapid exchange between the gases and liquids in the flasks.

After the manometers and flasks had been put into place with the stopcocks open, the shaking mechanism was run for one hour. At the highest and lowest temperatures used in the experiments, this period was extended to 2 hours. By this time the temperature in the flasks was equal to that of the water bath and practically all of the carbon dioxide originally present in the flasks had been absorbed. The stopcocks were then closed, and the level of the liquid in each manometer was recorded at intervals of one hour.

The volume of the pelomyxae was calculated by measuring specimens in a volumescope. This apparatus, devised by Chalkley (1929) and modified by Belda (1942), consists essentially of a capillary pyrex glass tube into which a pelomyxa can be put. The bore of the tube is such that the pelomyxa assumes a cylindrical shape with rounded ends. The length of the specimen can be measured accurately by means of a compound microscope provided with a camera lucida. The volume of the specimen can be calculated by using the equation

$$V = \pi r^2 l + 4/3\pi r^3$$

in which r is the radius, both of the cylindrical part of the pelomyxa and of each of the rounded ends, and l is the length of the cylindrical part.

The surface area of a pelomyxa varies considerably, depending on the number of pseudopodia which may be extended. The average surface area is, however, approximately equal to that of a specimen which has the shape of an elongated cylinder (Belda, 1943). Accordingly, the equation

$$A = 2\pi rl + 4\pi r^2$$

in which the symbols have the same value as in the preceding equation, is appropriate for calculating the approximate surface area.

In conducting the experiments, 20 specimens of average size were selected from those to be used in each test. These specimens were taken up with a capillary pipette and measured in the volumescope. At the conclusion of each test 20 specimens were again selected and measured. From these measurements the average volume and surface area was calculated. There was no appreciable variation between the average values obtained at the beginning and at the end of each experiment.

Results

I. Effect of food content on respiration

In much of the work previously reported on cellular respiration in the Protozoa, the food content of the organisms was not considered. However, Lund (1918) observed that there was a decrease in the rate of oxygen consumption in paramecia which had been starved for 20 hours, and Leichsenring (1925) observed a decrease of 23 per cent in the rate of oxygen consumption in paramecia which had been starved for 24 hours.

In order to ascertain what types of pelomyxae would be suitable for studying the effect of different temperatures on the rate of respiration, preliminary experiments were carried out on specimens in varying states of nutrition. The first tests were made with what are designated well-fed specimens. About 1000 pelomyxae together with numerous paramecia were put into finger-bowls containing buffer solution as described under Material and Methods. After 24 hours each pelomyxa had several large food vacuoles containing partly digested paramecia. Several hundred pelomyxae of average size were removed with a capillary pipette and washed in three separate portions of sterile buffer solution. Five milliliters of sterile buffer solution containing either 100 or 200 pelomyxae were put into each manometer flask; and 5 milliliters buffer solution without pelomyxae were put into the flask of the thermo-barometer. In the first three tests both oxygen consumption and carbon dioxide elimination were measured. The results are presented in Table II.

In the first three tests, the rate of oxygen consumption was 15,530 $mm.^3$, and of carbon dioxide elimination 13,510 $mm.^3$, per hour per million organisms. Calculated from these values, the respiratory quotient is 0.87.

For the second part of the experiment, pelomyxae were put into finger bowls containing buffer solution without paramecia and left for one week without food. These are designated starved pelomyxae. At the end of this time all food vacuoles have disappeared, and the average volume of the specimens is about 25 per cent less than that of well-fed pelomyxae. The average rate of oxygen consumption of

starved pelomyxae per million organisms was found to be about 65 per cent less than that of well-fed pelomyxae; however, the rate of oxygen consumption per mm.^{3} of cell substance showed a decrease of only 54 per cent. In the first three tests with starved pelomyxae, the average rate of carbon dioxide elimination per million organisms was 3,490 mm.^{3} per hour, a decrease of 73 per cent from that of well-fed specimens. The respiratory quotient was 0.56.

In the third part of the experiment, pelomyxae were put into finger bowls containing buffer solution, and large numbers of paramecia were added as in the first part of the experiments. After 3 days the pelomyxae had ingested nearly all the paramecia, but still contained numerous large food vacuoles. The pelomyxae were

TABLE II

Rate of oxygen consumption in starved, "normal," and well-fed specimens of Pelomyxa carolinensis
Temperature: 25° C. Hydrogen-ion concentration: pH 6.8

Type of organism	Test number	Number of organisms in each test	Duration of experiment	Average volume of one million organisms in mm.^{3}	Average rate of O_2 consumption in mm.^{3} per hour per million organisms	Average rate of O_2 consumption in mm.^{3} per hour per mm.^{3} of cell substance
Starved	1 to 3	100	5 hours	27,600	6,200	0.188±0.052
	4 to 6	200	6 hours		4,890	
	7 to 9	200	6 hours		4,340	
					Mean: 5,210±1,450	
"Normal"	1 to 3	100	5 hours	35,580	9,730	0.275±0.032
	4 to 6	200	7 hours		9,950	
	7 to 9	200	12 hours		9,730	
					Mean: 9,800±1,140	
Well-fed	1 to 3	200	5 hours	36,800	15,530	0.407±0.040
	4 to 6	200	7 hours		15,270	
	7 to 9	100	7 hours		14,120	
					Mean: 14,970±1,500	

left in the finger bowls for 3 more days, but no more paramecia were added. At the end of this time most of the organisms still had two or three small food vacuoles. These organisms are designated "normal" pelomyxae. The results of all these experiments are combined in Table II. This table shows that the rate of respiration in *Pelomyxa carolinensis* is closely correlated with the amount of food material present in the cytoplasm, for there is a progressive increase in the rate of oxygen consumption in starved, "normal," and well-fed specimens.

II. Effect of temperature on respiration

In the previous experiment, there was less variation in the rate of oxygen consumption of "normal" pelomyxae than of starved or well-fed specimens. Accordingly the remaining tests were all made with "normal" specimens.

A number of tests were made to measure the rate of oxygen consumption of

Pelomyxa at temperatures between 15° C. and 40° C., using 5° increments. Groups of either 100 or 200 organisms in 5 ml. buffer solution, treated as described above for "normal" pelomyxae, were tested. The results are given in Table III. This table shows that the rate of oxygen consumption for Peolmyxa increases in nearly

TABLE III

Rate of oxygen consumption in Pelomyxa carolinensis *at different temperatures. Hydrogen-ion concentration, pH 6.8*

Each figure under oxygen consumption per million organisms represents the average for three tests except those taken at 25° C. which are averages for four tests.

Temperature	Test number	Number of organisms used	Duration of experiment	Average volume of one million organisms in mm.³	Average approximate surface area/million organisms in mm.²	Average rate of O_2 consumption in mm.³ per hour per million organisms	Average rate of O_2 consumption in mm.³ per hour per mm.² cell substance
15° C.	1 to 3	200	7 hours	34,950	583,000	4,660	0.144±0.024
	4 to 6	200	7 hours			5,080	
	7 to 9	100	4 hours			5,280	
	10 to 12	200	5 hours			5,130	
						Mean = 5,040± 850	
20° C.	1 to 3	200	7 hours	36,800	607,900	7,070	0.191±0.022
	4 to 6	200	5 hours			6,920	
	7 to 9	100	5 hours			7,150	
						Mean = 7,050± 827	
25° C.	1 to 4	100	12 hours	36,800	607,900	10,250	0.244±0.028
	5 to 8	200	10 hours			8,750	
	9 to 12	200	6 hours			8,170	
	13 to 16	100	12 hours			8,540	
	17 to 20	200	10 hours			9,930	
	21 to 24	200	6 hours			8,380	
						Mean = 9,010± 910	
30° C.	1 to 3	200	12 hours	35,580	590,000	10,990	0.372±0.049
	4 to 6	100	12 hours			14,455	
	7 to 9	200	5 hours			14,420	
						Mean = 13,244±1,760	
35° C.	1 to 3	150	5 hours	34,950	583,000	19,010	0.507±0.044
	4 to 6	200	6 hours			17,530	
	7 to 9	100	6 hours			16,630	
						Mean = 17,749±1,540	

linear ratio from 15° to 25° C. Between 25° and 35° C. the rate of oxygen consumption increases more rapidly, but again in nearly linear ratio.

The lowest temperature at which tests were carried out was 15° C. At this temperature the pelomyxae showed a tendency to become spherical. The amount of protoplasmic streaming and the rate of locomotion were considerably diminished.

At the higher temperatures, the pelomyxae were more active. At 35° C. the rate of locomotion was rapid; long, narrow pseudopodia were extended in many directions, so that the organisms were nearly stellate in shape. All the specimens remained alive during these tests.

In nine of the 12 tests which were made at 40° C. the pelomyxae died during the first hour. In the remaining three tests, the organisms survived long enough for a single reading of the manometers to be made. The average rate of oxygen consumption for the three tests, each made with 100 pelomyxae at 40° C., was 28,300 $mm.^3$ per hour per million organisms. This rate is much higher than that at 35° C. Because of the high mortality of the organisms at 40° C., the results obtained are probably not comparable to those at lower temperatures, and they are not included in the table.

The temperature coefficient (Q_{10}) for the rate of oxygen consumption of Pelomyxa between 15° and 25° C. is 1.7; between 25° and 35° C. the Q_{10} is 2.1.

Discussion

The rate of oxygen consumption in well-fed pelomyxae is 2.16 times that of starved specimens. This difference is similar to that found by Lund (1918) in comparable types of Paramecium. He found that specimens tested after having been fed boiled yeast suspension used up from two to three times as much oxygen as specimens tested after having been starved.

The rate of oxygen consumption of pelomyxae kept for 7 days without food is 32 per cent less than that of "normal" specimens kept for 3 days with practically no food. Similarly, the rate of oxygen consumption of pelomyxae kept for 3 days with practically no food is 32 per cent less than that of well-fed pelomyxae. This difference is comparable to a decrease of 29 per cent in paramecia after having been kept for 72 hours without food, as measured by Leichsenring (1925). The close agreement between the results for Paramecium and Pelomyxa may, however, not be significant, since the two organisms differ greatly in their structure and activity.

The published data for respiration in Paramecium are based on the quantity of oxygen consumed and carbon dioxide given off per organism. They do not take into account the volume nor the surface area of the specimens. At least approximate values for the volume and surface area of Paramecium can be calculated by means of the equations given by Fortner (1925). These, however, require measurements of the length and diameter of the specimens.

That the volume of the organisms ought to be considered, at least in tests made on organisms differing in food content, can be seen from Table II. The decrease in the rate of oxygen consumption per unit of protoplasm for starved pelomyxae is relatively less than the decrease per organism.

Apparently the only measurement of the respiratory quotient of an amoebid organism previously reported is that of Emerson (1929), who found a value of slightly less than 1.0 for *Amoeba proteus*. However, the R.Q. for other free-living Protozoa has been measured. Emerson (1929) found a value of slightly less than 1.0 for *Blepharisma undulans*. The R.Q. for Paramecium, as found by various investigators, is as follows: Amberson (1928), 0.69; Root (1930), 0.62; and Mast, Pace, and Mast (1936), 0.72. These values are all similar to that of

Pelomyxa carolinensis. The significance of measurements of the R.Q. for various Protozoa has been discussed at length by Jahn (1941).

The rate of oxygen consumption per hour per mm.^{3} of protoplasm for Pelomyxa at 20° C., namely 0.191 mm.^{3}, is slightly higher than the rate of 0.16 mm.^{3} for *Amoeba proteus* found by Emerson (1929). Considering possible errors in the measurements of the volume of the organisms, this difference may not be significant. The rate of oxygen absorption per unit of surface area in *Amoeba proteus* is, however, much less than that in Pelomyxa. An exact comparison is not possible because Emerson did not measure the surface area of Amoeba. But since the volume of Amoeba is only about 1/50 that of Pelomyxa (Belda, 1942), the amount of surface area of Amoeba per mm.^{3} of protoplasm is many times greater than that of Pelomyxa.

Howland and Bernstein (1931) did not calculate the rate of oxygen consumption per unit volume of protoplasm in *Actinosphaerium eichhornii*. However, an approximate value can be obtained. The average diameter of *Actinosphaerium eichhornii* is between 200 and 300 micra. Since the organisms are approximately spherical in shape, the volume can readily be calculated from the diameter. The average rate of oxygen consumption at 20° C. is 0.26 mm.^{3} per mm.^{3} of protoplasm for specimens of Actinosphaerium having a diameter of 200 micra, and 0.11 mm.^{3} for specimens having a diameter of 300 micra. These values are of the same order of magnitude as those for Pelomyxa at 20° C.

The rate of oxygen consumption of Pelomyxa at 15° C. is 25 per cent less than that at 20° C. This difference is nearly equal to that observed in Paramecium by Leichsenring (1925). She measured the rate of respiration in paramecia at 20° C., then lowered the temperature to 15° C., and found that the rate of respiration had decreased 30 per cent.

Leichsenring also tested paramecia at 20° C., then increased the temperature to 35° C., and found an increase of 35 per cent in the rate of respiration. This is much less than the increase in rate found in the present investigation over the same range of temperature, since the rate of oxygen consumption in Pelomyxa is 135 per cent higher at 35° C. than at 20° C. It should be kept in mind that Leichsenring observed the effects of change in temperature on the same culture of Paramecium, whereas in the present experiment different lots of Pelomyxa were used at the different temperatures. However, Barratt (1905) found that the rate of CO_2 production of Paramecium at 27–30° C. was more than twice that at 15° C. This difference is nearly equal to that difference in the rate of oxygen consumption of Pelomyxa between these temperatures.

The Q_{10} value of 1.7 between 15° and 25° C. for pelomyxa is less than the Q_{10} value of 2.1 found by Lwoff (1933) for Paramecium between 13° and 23° C. On the other hand, the Q_{10} value of 2.1 for Pelomyxa between 25° and 35° C. is higher than the value of 1.5 for Paramecium between 23° and 32° C. found by Kalmus (1928) and also by Lwoff (1933).

Summary

1. The rate of respiration in *Peolmyxa carolinensis* at 25° C. is closely correlated with the amount of food material present in the cytoplasm.

2. The rate of oxygen consumption at 25° C. was found to be 0.244 ± 0.028

mm.^{3} per hour per mm.^{3} cell substance and does not differ greatly from that of *Amoeba proteus* and *Actinosphaerium eichhornii*. The rates were less at low temperatures (15° C.) and greater at high temperatures (35° C.).

3. Temperatures above 35° C. are usually lethal to Pelomyxa, although in several tests the oxygen consumption at 40° C. for one hour was obtained.

4. The respiratory quotient is much lower for starved specimens (R.Q.: 0.56) than for well-fed specimens (R.Q.: 0.87) and is approximately equal to that of other free-living Protozoa.

5. As the temperature is increased, the rate of oxygen consumption increases, but the rate of increase becomes progressively greater.

6. The temperature coefficient for the rate of respiration in *Pelomyxa carolinensis* is nearly the same as that in Paramecium, varying from 1.7 between 15° and 25° C. to 2.1 between 25° and 35° C.

LITERATURE CITED

Amberson, E. F., 1929. The influence of oxygen tension upon the respiration of unicellular organisms. *Biol. Bull.*, **55**: 79–91.

Barratt, J. O. W., 1905. Die Kohlensäureproduktion von Paramecium aurelia. *Z. allg. Physiol.*, **5**: 66–72.

Belda, W. H., 1942. Permeability to water in Pelomyxa carolinensis. I. Changes in volume of Pelomyxa carolinensis in solutions of different osmotic concentration. *The Salesianum*, **37**: 68–81.

Belda, W. H., 1943. Permeability to water in Peolmyxa carolinensis. III. The permeability constant for water in Pelomyxa carolinensis. *The Salesianum*, **38**: 17–24.

Chalkley, H. W., 1929. Changes in water content in Amoeba in relation to changes in its protoplasmic structure. *Physiol. Zool.*, **2**: 535–74.

Emerson, R., 1929. Measurements of the metabolism of two Protozoans. *Jour. Gen. Physiol.*, **13**: 143–158.

Fortner, H., 1925. Über die Gesetzmässigkeit der Wirkungen des osmotischen Druckes physiologisch indifferenter Lösungen aut einzellige, tierische Organismen. *Biol. Centralbl.*, **45**: 417–446.

Hahnert, W. F., 1932. Studies on the chemical needs of Amoeba proteus: a culture method. *Biol. Bull.*, **62**: 205–211.

Howland, R. B., and A. Bernstein, 1931. A method of determining the oxygen consumption of a single cell. *Jour. Gen. Physiol.*, **14**: 339–348; 352–403.

Jahn, T. L., 1941. Chapter VI. Respiratory metabolism. *Protozoa in Biological Research*. Columbia Univ. Press.

Kalmus, H., 1928. Die Messung der Atmung, Gärung, und CO_2-Assimilation kleiner Organismen in der Kapilläre. *Zeit. vergl. Physiol.*, **7**: 304–313.

Leichsenring, J. M., 1925. Factors influencing the rate of oxygen consumption in unicellular organisms. *Amer. Jour. Physiol.*, **75**: 84–92.

Lund, E. J., 1918. III. Intracellular respiration, relation of the state of nutrition of Paramecium to the rate of intracellular oxidation. *Amer. Jour. Physiol.*, **47**: 167–177.

Lwoff, A., 1933. Die Bedeutung Des Blutfarbstoffes für die parasitischen Flagellaten. *Zbl. Bakt. Abt.* 1 (Orig.), **130**: 498–518.

Mast, S. O., D. M. Pace, and L. R. Mast, 1936. The effect of sulfur on the rate of respiration and on the respiratory quotient of *Chilomonas paramecium*. *Jour. Cell. and Comp. Physiol.*, **8**: 125–139.

Root, W. S., 1930. The influence of carbon dioxide upon the oxygen consumption of Paramecium and the egg of Arbacia. *Biol. Bull.*, **59**: 48–69.

THE EFFECT OF TEMPERATURE ON THE RATE OF DISAPPEARANCE OF CAUDAL BANDS IN FUNDULUS HETEROCLITUS [1, 2]

FREDERICK P. FERGUSON [3]

(Department of Zoology, University of Minnesota, and the Marine Biological Laboratory, Woods Hole)

Introduction

It has been known since the work of Wyman (1924) that a transverse cut made near the base of the caudal fin of the killifish, *Fundulus heteroclitus* Linn., severs the nerve supply to the melanophores in the area posterior to the incision. In a white-adapted fish, this operation results in the immediate appearance of a sharply defined black band of completely dispersed melanophores, extending distally from the cut to the posterior edge of the tail.

From the experiments of Mills (1932a) and Parker (1934a) evidence has been accumulated for the hypothesis that the formation of the band is due to the mechanical stimulation, by cutting, of the melanophore-dispersing nerves to this region of the tail. Moreover, the tendency of such a band to be maintained over a period of one or more days is believed to be due to the prolonged though gradually diminishing activity of the momentarily stimulated dispersing fibers (Parker, 1934a).

If a fish with a caudal band is allowed to remain upon a white illuminated background, however, the stripe gradually blanches, the process starting at the edges and progressing inward toward the central region as an increasing number of melanophores concentrate their pigment to assume the appearance of those in normally innervated parts of the tail. This sequence of fading was first reported by Mills (1932b), who regarded it as evidence for the hypothesis that the blanching of caudal bands is due to a concentrating neurohumor secreted by the terminals of concentrating nerves in adjacent regions of the tail and capable of diffusing through the tissues into the denervated area to produce its characteristic effect upon the dispersed melanophores. This hypothesis has been actively supported by several investigators during the past decade, and is now quite generally accepted as the explanation for the blanching of caudal bands in Fundulus and in other teleosts.

The following investigation was undertaken in an attempt to determine the effect of temperature on the rate of disappearance of these caudal bands in Fundulus. A brief study of this type has been made upon the normal paradise fish, *Macropodus opercularis,* by Dalton and Goodrich (1937). These authors found that in each of four animals disappearance of the band required about one half the

[1] Adapted from a section of a thesis submitted to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Aided by grants from the Charles Peter Sigerfoos Fellowship Fund in Zoology.

[3] Present address: Department of Physiology, Louisiana State University School of Medicine, New Orleans 13, La.

time at 29° C. that it did at 20° C.[4] No other experiments of this sort have been encountered in the literature.

Material and Methods

The experiments to be reported were performed upon *Fundulus heteroclitus*. Its hardiness and availability, together with its conspicuous melanophore responses, made it an especially favorable object for the type of studies involved. Preliminary observations soon made it apparent that the more uniform size and distribution of caudal melanophores in females facilitated the accurate determination of the disappearance of caudal bands, and consequently they were employed exclusively throughout the investigation.

The fish were seined, trapped, or caught with dip nets in the brackish waters in the area of Woods Hole. Healthy females, three to four inches in length, with undamaged caudal fins were then brought to the laboratory where they were used immediately or stored temporarily in a large sink provided with running sea water. This stock of fish, as well as those used in experiments involving a period of several days, was fed every other day on the meat of the clam, *Venus mercenaria* or the edible mussel, *Mytelus edulis*.

Two constant temperature baths were employed in controlling the temperature of the water in which the fish were kept during the experiments. These baths were copper-lined tanks with capacities of approximately 5 cubic feet. Each was filled with tap water which was kept in continuous circulation to insure uniformity of temperature throughout the bath. The temperature of this water was controlled by competition between the cooling effect of a regulated stream of ice water or tap water (depending upon the temperature to be maintained) and the heating effect of a submerged resistance unit controlled by a mercury-toluene thermoregulator through a vacuum tube relay operating on A. C. With proper adjustment of the thermoregulator and the incoming stream of water, it was possible to maintain the temperature of the baths to within extreme limits of $\pm$ 0.5° C. of the desired level over the range employed in these experiments.

A wire shelf suspended 7 inches below the water level in each bath provided support for three flat-bottomed aquaria, 10 inches in diameter and 8 inches in height, which were painted white on the outside and filled with sea water to the level of the water in the bath. Each of these served as a container for four or five fish during the experiments and was aerated by a gentle stream of washed air from a small electric compressor. Illumination was provided by a 60-watt electric light bulb equipped with a white reflector and suspended 15 inches above the surface of the water of each bath. Several trial experiments were performed to determine the advisability of covering each bath with a black tent to eliminate diurnal fluctuations of light intensity. This precaution was found to be unnecessary. Moreover such an arrangement increased the difficulty of maintaining the lower temperatures, and consequently was not employed.

Caudal bands were produced according to the method of Wyman (1924), by making a transverse cut of appropriate length at the base of the tail, approximately one millimeter posterior to the last row of scales. Each animal was allowed to

[4] When calculated from their data, the mean Q_{10} for the group is found to be 2.27.

adapt itself completely to the white background and to the temperature at which the experiment was to be run. The head and trunk were then carefully wrapped in moist cheese cloth and the animal was laid on its right side in a large Petri dish filled with white paraffin. The protruding caudal fin was spread slightly and held flat against the paraffin by the operator's left forefinger while the cut was made. "One-ray bands" were produced by cuts one mm. in length, which severed a single fin ray and approximately half of each adjacent interray. "Two-ray bands" were produced by cuts twice this length, and included two rays, the interray between them, and approximately half of each interray bounding their outer sides. As already noted by many investigators, the large majority of these operations are unaccompanied by any disturbance of blood supply to the denervated area except for the region immediately surrounding the cut.

After the operation each fish was carefully measured from the tip of its snount to the conspicuous arc of blood vessels (Fries, 1931) at the base of the tail. The length to the nearest millimeter was then recorded, together with a sketch indicating the shape and other characteristics of the tail and the position of the band. Such a procedure proved very valuable for insuring future identification of the individual.

After this treatment the fish was returned to its white aquarium in the constant temperature bath, where the band was allowed to fade. The early progress of fading was followed by macroscopic observations at various intervals. Unhealthy animals as well as those whose tails became injured in any way to affect the band were always discarded as soon as they were noticed. When the band became rather faint, subsequent observations were made in so far as possible at intervals of two hours. More frequent observations were generally impractical because of the injurious effects of excessive handling and inability to perceive definite changes in the state of most bands over intervals of less than two hours.

Final stages of blanching were always observed with the aid of a binocular dissecting microscope (10X oculars, 32 or 48 mm. objectives), a method which was found to be more reliable than the use of the unaided eye. Since the blanching is a gradual process, it is of course impossible to determine the exact end point by observations made at intervals of time. Consequently, the mid-point between the time of the last observation in which pigment dispersion was manifested by any of the band melanophores and the first one in which all of these cells had concentrated their pigment as completely as those in the normally innervated regions of the tail was taken as the best estimate of the time of complete fading. Thus, if a band were still very slightly evident 24 hours after its formation, but had completely disappeared by the time of the next observation two hours later, the blanching time would be recorded as 25 hours. Confirmatory observations were then continued for several hours.

Results

Preliminary. Before proceeding with experiments on the relation of temperature to the rate of disappearance of caudal bands, studies were made to determine whether or not an individual Fundulus manifests a more or less characteristic reaction time at a particular temperature level. The advisability of such a study was emphasized by the fact that a great deal of variation had been found to occur among different individuals under the same environmental conditions. Thus, for instance,

in a group of 52 Fundulus with one-ray bands the blanching time at 20° C. varied from 16 to 197 hours; in a group of 17 at 25°, it varied from 13 to 138 hours; and in a third group of 48 at 30°, it varied from 4 to 66 hours.

In determining the degree of constancy of blanching time for an individual, a single one-ray band was produced and its time of disappearance recorded. The fish was then allowed to remain in its white aquarium, and after a few hours a second band was made by cutting another ray a few millimeters above or below the faded area. The time required for disappearance of this second band was then determined without reference to that required by the first one.

It was found that, with few exceptions, the time required for the disappearance of the two bands was remarkably similar. In the majority of cases the difference lay between zero and 2 hours. For a group of 25 fish at 30° C., the mean difference in time of disappearance of the first and second bands was 0.54 ± 1.07 [5] hours. From these data the significance of the mean difference can be estimated from the probability value (P) corresponding to the familiar "t" criterion.[6] For this experiment $t = 0.505$, corresponding to a P value of something over 0.55. Thus, the chances of such a difference being exceeded through errors of random sampling are greater than 55 per cent, and consequently it cannot be regarded as significant. Similar results were obtained with smaller numbers of fish at 15°, 20°, and 25° C. and it was therefore concluded that at a given temperature level there is no significant difference in the blanching time of two one-ray caudal bands produced at different times upon the same fish.

It might be of interest to note that one set of determinations was carried out in such a way as to compare the time of blanching of one-ray bands in sea water and in fresh water at 30° C. During the fading of the first band the fish were kept in sea water, but this was replaced by ordinary tap water for the second run. Of eight individuals successfully carried through the experiment, the mean difference in blanching time of the two bands was 0.44 ± 0.63 hours. The probability of such a difference being exceeded through errors of random sampling was found to be 0.5076 (50.76 per cent) and it is therefore apparent that the rate of disappearance of bands in fresh water is not significantly different from that in sea water.

Temperature. With these preliminary studies in mind, the problem of determining the effect of temperature on the rate of the fading reaction was undertaken. In carrying out these experiments, the time required for blanching at one temperature was determined as usual, and the fish was then transferred to the second bath, the temperature of which was regulated at 10° C. higher or lower than that of the

[5] Standard error of mean. In this paper, standard deviation has been calculated from the formula $s_x = \sqrt{\frac{\Sigma x^2}{N} - \bar{x}^2}$ (Treloar, 1939, p. 55), in which s_x represents the standard deviation; Σx^2 the sum of the squares of each variate; $\bar{x}^2$ the square of the mean; and N the number of individuals in the series. The standard error of the mean has been calculated as $\text{S.E.}_{\bar{x}} = \frac{s_x}{\sqrt{N-1}}$ (Treloar, 1939, p. 138).

[6] Calculated from the equation $t = \frac{\bar{d}}{\text{S.E.}_{\bar{d}}}$ (Treloar, 1942, p. 54), in which $\bar{d}$ represents the mean difference between the pairs of values, and $\text{S.E.}_{\bar{d}}$ the standard error of that mean difference. Corresponding values of P were obtained from Treloar's (1936) extensive table of probability in terms of t.

first. The animal was allowed to adapt itself gradually to the new temperature. After a few hours a new band was produced above or below the site of the original and the time of its disappearance determined. In view of the preliminary work described above, any significant difference in the time required for disappearance of the two bands could be reasonably ascribed to the change in temperature.

Table I shows the results obtained in 17 successful cases in which the first band was allowed to fade at 15° and the second at 25° C. In the column under the

TABLE I

Time in hours required for blanching of one-ray caudal bands at 15° and at 25° C. on the same fish. First run at 15°, second at 25°

Range, interval during which final blanching occurred; Mp, mid-point of that interval; S.D., standard deviation; S.E., standard error of mean.

Fish	Run 1: 15°		Run 2: 25°		Difference $Mp_{15}-Mp_{25}$ (hrs.)	Q_{10}
	Range (hrs.)	Mp_{15} (hrs.)	Range (hrs.)	Mp_{25} (hrs.)		
1	88–90	89	62–66	64	25	1.39
2	74–76	75	42–44	43	32	1.74
3	44–46	45	22–24	23	22	1.96
4	114–116	115	56–58	57	58	2.02
5	44–46	45	20–22	21	24	2.14
6	268–276	272	124–126	125	147	2.18
7	98–100	99	38–48	43	56	2.30
8	98–100	99	38–48	43	56	2.30
9	190–192	191	66–68	67	124	2.85
10	172–182	177	54–56	55	122	3.22
11	170–172	171	52–54	53	118	3.23
12	76–78	77	18–24	21	56	3.67
13	184–186	185	46–48	47	138	3.94
14	88–90	89	20–22	21	68	4.24
15	182–186	184	40–42	41	143	4.49
16	212–216	214	46–48	47	167	4.55
17	146–148	147	26–28	27	120	5.44
Mean	133.76		46.94		*86.82	3.04
S.E.	±15.65 hrs.		±6.08 hrs.		±12.06 hrs.	±0.285
S.D.	62.61 hrs.		24.32 hrs.		48.23 hrs.	1.14

* $t = 7.20$; $P = <.0001$.

heading "Fish" the animals have been arranged arbitrarily in order of increasing values of Q_{10}, recorded in the last column. It can be seen that in every case the blanching reaction required much less time at the higher temperature, the difference being designated in the appropriate column. From 133.76 ± 15.65 hours at 15° the mean time required decreased to 46.94 ± 6.08 hours at 25° C. The value of t calculated from the mean difference is beyond the tabulated range, indicating a probability of less than 1 : 10,000 that such a difference would be exceeded through errors of random sampling.

More informative than the actual difference in time required at the two tem-

perature levels, however, are the Q_{10} values given in the last column of the table. These were found to vary about a mean of 3.04 ± 0.285, indicating that on the average the rate of blanching of caudal bands was approximately trebled by increasing the temperature from 15° to 25° C.

The results obtained upon 14 fish in which the first one-ray band was allowed to fade at 20° and the second at 30° C. are shown in Table II. Here again the

TABLE II

Time in hours required for blanching of one-ray caudal bands at 20° and at 30° C. on the same fish. First run at 20°, second at 30°

Range, interval during which final blanching occurred; Mp, mid-point of that interval; S.D., standard deviation; S.E., standard error of mean.

Fish	Run 1: 20°		Run 2: 30°		Difference $Mp_{20}-Mp_{30}$ (hrs.)	Q_{10}
	Range (hrs.)	Mp_{20} (hrs.)	Range (hrs.)	Mp_{30} (hrs.)		
1	35–36	35.5	22–24	23	12.5	1.54
2	36–44	40	24–26	25	15	1.60
3	39–41	40	21–23	22	18	1.82
4	37–39	38	19–21	20	18	1.90
5	84–86	85	38–48	43	42	1.98
6	36–40	38	18–20	19	19	2.00
7	43–45	44	21–23	22	22	2.00
8	51–55	53	25–27	26	27	2.04
9	22–24	23	10–12	11	12	2.09
10	72–74	73	34–36	35	38	2.09
11	78–80	79	36–38	37	42	2.14
12	46–48	47	20–22	21	26	2.24
13	84–93	88.5	26–28	27	61.5	3.28
14	56–58	57	14–16	15	42	3.80
Mean [7]	52.93		24.71		*28.21	2.18
S.E.	±5.48 hrs.		±2.30 hrs.		±3.93 hrs.	±0.166
S.D.	19.79 hrs.		8.32 hrs.		14.17 hrs.	0.60

[7] Discrepancy of 0.01 between difference of means and mean difference due to rounding off figures at second decimal place.

* $t = 7.18$; $P = <.0001$.

rise of 10° produced a marked increase in the rate of the reaction in every case, the mean Q_{10} value being 2.18 ± 0.166.

A similar relation of temperature to the rate of fading was found in a series of seven Fundulus in which one two-ray band was allowed to disappear at 20° and the second at 30° C. The mean time required at the lower temperature was 126.79 ± 11.31 hours, whereas that at the higher level was 63.29 ± 8.24 hours, the mean difference being 63.50 ± 6.14 hours. It will be noted that the blanching time of these bands at each temperature was much longer than that of the one-ray bands at the same level (cf. Parker, 1934b). The Q_{10} values varied about a mean of 2.09 ± 0.16, however, indicating that in these bands, as in the narrower ones, fad-

ing occurs approximately twice as rapidly when the temperature is raised from 20° to 30° C.

Table III shows the results of another experiment which was also carried out at 20° and at 30° C., but with the temperature sequence reversed. In this case the first determination of blanching time was made at 30° and the fish were then

TABLE III

Time in hours required for blanching of one-ray caudal bands at 20° and at 30° C. on the same fish. First run at 30°, second at 20°

Range, interval during which final blanching occurred; Mp, mid-point of that interval; S.D., standard deviation; S.E., standard error of mean.

Fish	Run 1: 30°		Run 2: 20°		Difference $Mp_{30}-Mp_{20}$ (hrs.)	Q_{10}
	Range (hrs.)	Mp_{30} (hrs.)	Range (hrs.)	Mp_{20} (hrs.)		
1	15–17	16	24–26	25	9	1.56
2	23–24	23.5	36–48	42	18.5	1.79
3	24–26	25	46–50	48	23	1.92
4	31–33	32	62–64	63	31	1.97
5	23–24	23.5	46–48	47	23.5	2.00
6	23–25	24	47–49	48	24	2.00
7	23–24	23.5	47–49	48	24.5	2.04
8	51–53	52	108–120	114	62	2.19
9	18–20	19	41–43	42	23	2.21
10	17–19	18	40–42	41	23	2.28
11	27–31	29	65–72	68.5	39.5	2.36
12	23–24	23.5	55–57	56	32.5	2.38
13	22–24	23	52–62	57	34	2.48
14	14–23	18.5	48–50	49	30.5	2.65
15	15–17	16	42–44	43	27	2.69
16	25–27	26	70–72	71	45	2.73
17	25–27	26	73–75	74	48	2.85
18	15–17	16	46–48	47	31	2.94
19	13–15	14	43–45	44	30	3.14
20	25–29	27	87–91	89	62	3.30
21	25–29	27	98–109	103.5	76.5	3.83
22	11–13	12	46–50	48	36	4.00
23	13–14	13.5	55–57	56	42.5	4.15
Mean	22.96		57.57		*34.61	2.59
S.E.	±1.73 hrs.		±4.39 hrs.		±3.31 hrs.	±0.14
S.D.	8.10 hrs.		20.59 hrs.		15.34 hrs.	0.67

* $t = 10.46$; $P = <.0001$.

transferred to the lower temperature. The results were in all respects similar to those of the preceding experiments, a much longer time being required by each fish for the disappearance of the band at the lower temperature. From a consideration of the Q_{10} values it can be seen that in the majority of cases the rate of the reaction was more than twice as high at the 30-degree level as at 20°, the mean ratio being 2.59 ± 0.14.

Discussion

In *Fundulus heteroclitus*, the time required for the complete blanching of caudal bands of equal width varies greatly among different individuals, even when maintained under identical environmental conditions during the process. This variation was apparent in the preliminary studies of this investigation and was confirmed by all subsequent work. On the other hand, each fish manifests a characteristic reaction time for the process. Thus, two bands of equal width produced upon the same individual were found to fade at remarkably similar rates under the same environmental conditions, the difference in time required for their disappearance being of no statistical significance.

This characteristic reaction time for individuals makes it possible, by performing "paired" experiments upon each of a number of animals, to determine with considerable accuracy the relation of a particular variable factor to the time required for the fading of caudal bands in Fundulus. In this way one band of each animal serves in effect as a control for the other and any significant difference between the blanching time of the two can be reasonably attributed to the factor which has been varied. This method was employed in the present investigation to determine the effect of temperature upon the rate of the fading reaction.

The rate of fading of caudal bands in Fundulus was found to be influenced directly by temperature, a rise of 10° over the intervals 15 — 25° C. or 20 — 30° C. resulting in an average increase of 2 to 3 times in the speed of the reaction. The results obtained in this investigation thus agree in this respect with those of Dalton and Goodrich (1937) on the paradise fish, *Macropodus opercularis*. These authors ascribed their results to the effects of temperature upon the rate of diffusion of the concentrating neurohumor into the band from adjacent regions of the tail. This interpretation would admittedly add considerable significance to the immediate value of results of this sort in the light of the neurohumoral hypothesis. Unfortunately, however, such a conclusion is difficult to reconcile with the prevalent concept that the effect of temperature changes upon the rate of diffusion processes is ordinarily of considerably less magnitude than the effect observed upon the rate of disappearance of caudal bands in their study and in the present investigation. Jacobs (1928), for instance, states that ". . . processes whose rate is limited by the diffusion of dissolved substances (give) values of Q_{10} in the vicinity of 1.25 or 1.3; and chemical reactions of the ordinary sort values which are usually in excess of 2."

Hence, there is no *a priori* reason to regard the blanching reaction as one limited by diffusion, since its temperature coefficient falls within the range generally attributed to chemical reactions. This is not meant to imply, however, that diffusion is to be excluded from the total reaction. The blanching of caudal bands is undoubtedly a complex phenomenon, involving many different processes, several or possibly all of which may be affected by temperature. Consequently, the observed results may well represent the net effect of temperature upon the rate of the reaction as a whole rather than its influence upon any single process involved. It therefore seems advisable to regard the temperature coefficient of this reaction, like that of many other complex biological reactions, as being primarily of descriptive rather than of analytical value.

Summary

1. In *Fundulus heteroclitus*, the time required for the blanching of caudal bands of equal width varies greatly among different individuals under the same environmental conditions.

2. Each fish manifests a characteristic reaction time, however, and it is therefore possible, by performing "paired" experiments, to determine the effect of certain variable factors upon the rate of the blanching reaction in any individual.

3. By this method it has been found that the rate of blanching of caudal bands in Fundulus is directly influenced by temperature. A rise of 10° over the interval of 15° to 25° C. increased the speed of the reaction by an average of 3.04 ± 0.285 times in a group of 17 fish with one-ray bands. A similar rise over the interval of 20° to 30° C. increased it by an average of 2.18 ± 0.166 times in a group of 14 animals with one-ray bands, and 2.09 ± 0.16 times in seven animals with two-ray bands. Conversely, a decrease from 30° to 20° C. decreased the rate by an average of 2.59 ± 0.14 times in a group of 23 fish with one-ray bands.

It is suggested that these results be ascribed to the net effect of temperature upon the rate of the blanching reaction as a whole rather than to its influence upon any single process involved.

4. By the same method it was also found that the rate of blanching of caudal bands in fresh water is the same as that required in sea water. For a group of 8 animals with one-ray bands, the mean difference in reaction time in these two media at 30° C. was 0.44 ± 0.63 hours.

Acknowledgment

It is a pleasure to express my gratitude to Professor H. B. Goodrich of Wesleyan University and to Professor D. E. Minnich of the University of Minnesota for many kindnesses extended to me throughout the pursuit of this investigation and for helpful criticism of the manuscript.

LITERATURE CITED

Dalton, H. C., and H. B. Goodrich, 1937. Chromatophore reactions in the normal and albino paradise fish, Macropodus opercularis L. *Biol. Bull.*, **73**: 535–541.

Fries, E. F. B., 1931. Color changes in Fundulus, with special consideration of the xanthophores. *Jour. Exp. Zool.*, **60**: 389–426.

Jacobs, M. H., 1928. The complex nature of the effects of temperature on the rates of certain biological processes. *Amer. Nat.*, **62**: 289–297.

Mills, S. M., 1932a. The double innervation of fish melanophores. *Jour. Exp. Zool.*, **64**: 231–244.

Mills, S. M., 1932b. Evidence for a neurohumoral control of fish melanophores. *Jour. Exp. Zool.*, **64**: 245–255.

Parker, G. H., 1934a. The prolonged activity of momentarily stimulated nerves. *Proc. Nat. Acad. Sci. Washington*, **20**: 306–310.

Parker, G. H., 1934b. Cellular transfer of substances, especially neurohumors. *Jour. Exp. Biol.*, **11**: 81–88.

Treloar, A. E., 1936. *An outline of biometric analysis.* Burgess Pub. Co., Minneapolis. 193 pp.

Treloar, A. E., 1939. *Elements of statistical reasoning.* John Wiley and Sons, Inc., New York. 261 pp.

Treloar, A. E., 1942. *Random sampling distributions.* Burgess Pub. Co., Minneapolis. 94 pp.

Wyman, L. C., 1924. Blood and nerve as controlling agents in the movements of melanophores. *Jour. Exp. Zool.*, **39**: 73–132.

THE CHROMATIN IN THE LIVING ARBACIA PUNCTULATA EGG, AND THE CYTOPLASM OF THE CENTRIFUGED EGG AS PHOTOGRAPHED BY ULTRA-VIOLET LIGHT

ETHEL BROWNE HARVEY AND GEORGE I. LAVIN

(The Marine Biological Laboratory, Woods Hole; the Biological Laboratory, Princeton University; and the Rockefeller Institute for Medical Research, New York City)

CHROMATIN

In the living egg of *Arbacia punctulata*, as observed with high magnification with the best optical equipment for visible light, the mature nucleus in the unfertilized and recently fertilized egg appears clear and homogeneous. This is true in the normal egg where the red pigment somewhat obscures the picture, in the centrifuged whole egg where the pigment has been thrown down and the nucleus lies in the clear layer, and also in the white "half" (containing no pigment) obtained by centrifugal force. Nor can the chromosomes of the mitotic figure be seen in the living egg of Arbacia though the asters are very conspicuous. In sections of eggs, however, which have been fixed in Bouin's fluid and stained with Heidenhain's haematoxylin, the chromatin material is darkly stained and shows a characteristic structure, a network in the nucleus of the unfertilized and recently fertilized egg, and discreet bodies, chromosomes, during the mitotic divisions (Harvey, 1940).

A study of the chromatin material in the living egg of Arbacia as it appears with ultra-violet light has been made by the use of the quartz microscope devised by Köhler (1904), and modified by Lavin (1943). This is a microscope with quartz oculars and objectives so arranged that an accurate focus can be obtained on a fluorescent plate. The light source is a quartz spiral mercury resonance lamp (Hanovia Chemical Co.) from which the visible light is taken out by a liquid filter, so that only light of the wave length of 2537 A° is transmitted. The eggs were mounted on a quartz slide and covered with a quartz coverslip and partially compressed to make the eggs thin enough for the light to penetrate. Photographs of a selected field were taken with an exposure of three minutes. That the eggs were still living and coagulation had not set in was shown by progressive changes in serial photographs taken at intervals, of the same field of fertilized eggs.

We have arranged on Plate I photographs of similar stages of the egg of Arbacia: (1) Living eggs taken with ordinary visible light (Photographs 1, 4, 7). (2) Sections of eggs fixed in Bouin and stained with Heidenhain's haematoxylin, photographed with visible light (Photographs 2, 5, 8). (3) Living eggs photographed with ultra-violet light (Photographs 3, 6, 9). Three stages are shown, one of the immature egg with large germinal vesicle and nucleolus, a second of the mature egg with small nucleus, and a third of a late prophase or early metaphase. It will be seen that the chromatin material which does not show at all with visible light in the living egg is quite plain with the ultra-violet light and similar in appearance to the stained preparations with visible light.

The dark areas in the prints are, of course, the areas where the ultra-violet light is absorbed. The absorption is probably due to the presence of nucleic acid compounds since the purine and pyrimidine constituents of nucleic acid have an absorption maximum in this region of the spectrum. Of course this does not rule out the absorption due to the possible presence of proteins, but since the absorption of nucleic acid is much greater than that of the proteins, and until it is possible to differentiate between the two, the absorbing material will be referred to as nucleic acid compounds. It is not possible from the photographs to tell whether the absorbing materials are nucleic acids of the desoxyribose type (thymonucleic) or of the ribose type (yeast nucleic). The absorbing material in the immature egg (Photograph 3) is the nucleolus and a coarse network throughout the germinal vesicle; the bulk of the material in the germinal vesicle is non-absorbing, much less absorbing than the cytoplasm. In the mature nucleus (Photograph 6) the absorbing material is the chromatin network, and the rest of the material is less absorbing than the cytoplasm. In the dividing cell (Photograph 9) the chromosomes absorb and there is a considerable amount of non-absorbing material around them. There is no evidence of spindle or asters, such as one sees with visible light (Photographs 7, 8). There is the bare possibility that this may be due to the cell being somewhat compressed in order to allow the light to penetrate. The nucleic acid compounds of the nucleus, therefore, seem to be restricted to the nucleolus and network of the germinal vesicle of the immature egg, the chromatin threads of the mature nucleus and the chromosomes of the dividing egg.

It is generally agreed that the nucleic acid of the nucleus is of the desoxyribose type (thymonucleic) and that of the cytoplasm of the ribose type (yeast nucleic acid). The nucleolus is believed to be of the ribose type (Caspersson and Schultz,

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PLATE I

Photomicrographs, with approximate magnifications, as indicated, to bring comparative photographs to about the same size. Allowance is thus made for shrinking in fixation and expansion due to pressure of the coverslip for the ultra-violet.

PHOTOGRAPH 1. Living immature egg with visible light. 500 ×.

PHOTOGRAPH 2. Section of immature egg, fixed in Bouin and stained with Heidenhain's haematoxylin. Visible light. 660 ×.

PHOTOGRAPH 3. Living immature egg with ultra-violet light. 500 ×.

PHOTOGRAPH 4. Living mature unfertilized egg with visible light. 500 ×.

PHOTOGRAPH 5. Section of mature unfertilized egg, fixed in Bouin and stained with Heidenhain's haematoxylin. Visible light. 660 ×.

PHOTOGRAPH 6. Living mature unfertilized egg with ultra-violet light. 500 ×.

PHOTOGRAPH 7. Living fertilized egg at metaphase. Visible light. 500 ×.

PHOTOGRAPH 8. Section of fertilized egg at metaphase (early), fixed in Bouin and stained with Heidenhain's haematoxylin. 660 ×.

PHOTOGRAPH 9. Living fertilized egg at metaphase (early) with ultra-violet light. Egg somewhat squashed. 500 ×.

PHOTOGRAPH 10. Living egg centrifuged, 10,000 × g. for two minutes. Oil cap, clear layer, mitochondrial band, yolk, pigment. Nucleus under the oil cap. Visible light. 280 ×.

PHOTOGRAPH 11. Section of centrifuged egg, fixed in formalin and unstained. Visible light. 400 ×.

PHOTOGRAPH 12. Section of centrifuged eggs, fixed in formalin and stained with Heidenhain's haematoxylin. Visible light. 400 ×.

PHOTOGRAPH 13. Section of centrifuged eggs, fixed in formalin, and unstained. Ultra-violet light. 400 ×.

Visible; living except 11 Visible; stained sections Ultra-violet; living except 13

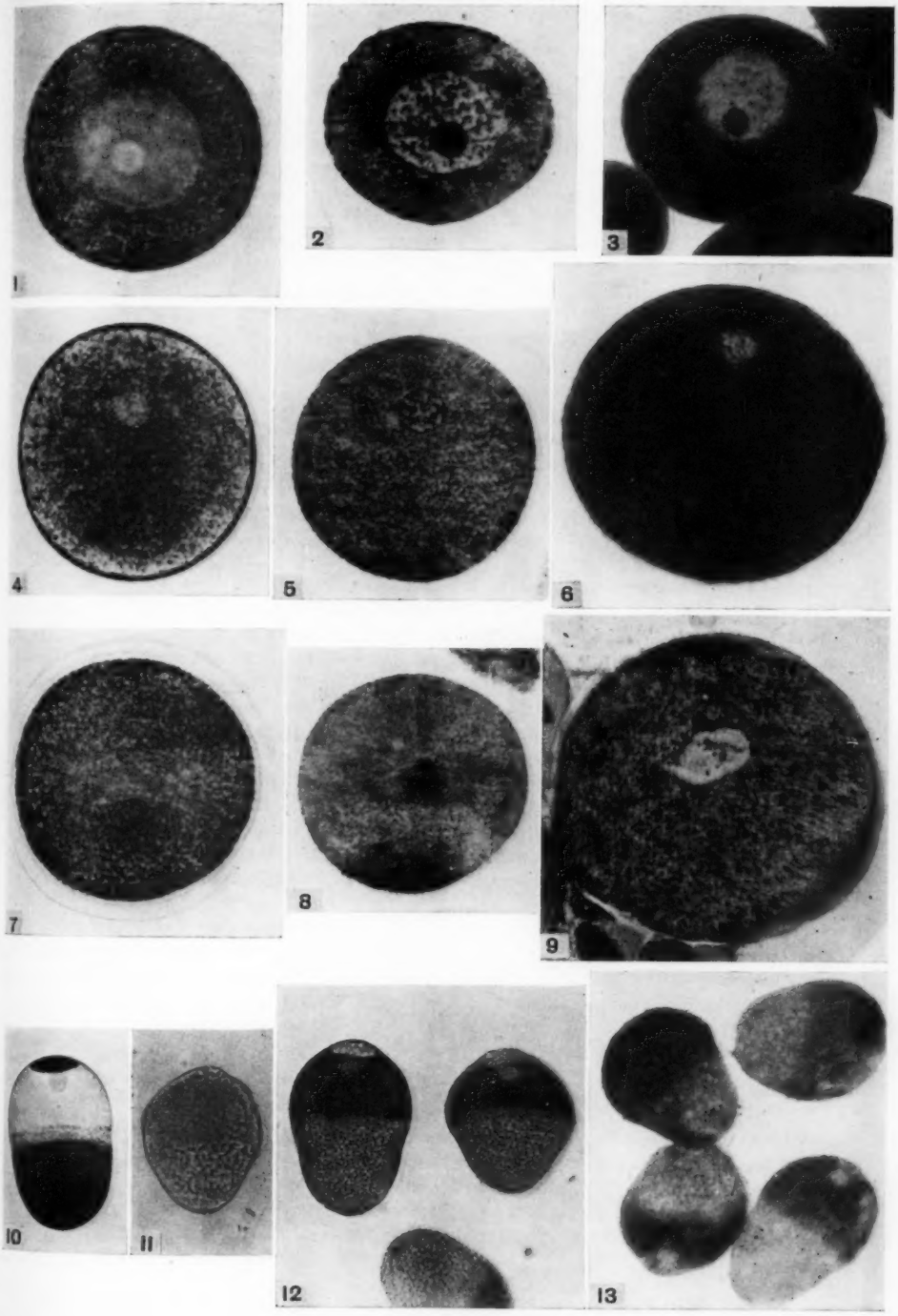

PLATE I

1940). The spindle of the dividing cell probably does not contain nucleic acids of either type, though there is some disagreement about this (Stedman and Stedman, 1943a, b, c vs. Callan, 1943; Barber and Callan, 1944).

Blanchard (1935) extracted from masses of unfertilized mature Arbacia eggs nucleic acid of the desoxyribose type and also a pentose derivative whose properties resembled those of a ribose nucleic acid, both in approximately the same amount. Brachet (1933, 1937) found little of the desoxyribose type in unfertilized eggs of another sea urchin, *Paracentrotus lividus*, and thought that Blanchard's results were due to the presence of ovarian tissue, other than the eggs themselves, rich in the desoxyribose type. Brachet found that the desoxyribose nucleic acid increased in amount and the ribose type decreased after fertilization. According to Caspersson and Schultz (1940), there is an accumulation of ribonucleic acid in the cytoplasm close to the nuclear membrane in the ovarian (immature) egg of *Psammechinus miliaris*, another sea urchin; this they think indicates a "synthesis of nuclear products influencing cytoplasmic activity." In our ultra-violet photographs of Arbacia, one cannot distinguish between the ribose and desoxyribose nucleic acids since they absorb equally. In some of the photographs there is a distinct massing of strongly absorbing material, either as a clump or as a thick ring, on the outside of the nuclear membrane, but it is not constant.

The first work on photography with ultra-violet light on living and fixed (unstained) cells was done by Köhler in 1904 with his original ultra-violet microscope. His photographs show very nicely the nuclei in living cartilage cells of Triton and the spireme threads and chromosomes of fixed epithelial cells of Salamandra. Very good ultraviolet photographs of grasshopper (*Melanoplus femur rubrum*) spermatocyte cells, showing spiremes and chromosomes in all stages of mitosis, were published by Lucas and Stark in 1931; they show no spindle fibers, though they state that these can be seen in unstained sections. Wyckoff and Ebeling (1933) show similar ultra-violet photographs of "grasshopper" spermatocytes. Caspersson (1936) shows similar photographs of such cells in other species of Orthoptera (*Chorthippus dorsatus* and *Gomphocerus maculatus*). These chromatic structures in Orthopteran sperm cells, however, show well in the living material with visible light (Chambers, 1914, in *Disosteira carolina;* Bělař, 1929, in *Chorthippus lineatus*). It is of interest that the giant chromosomes of the salivary gland cells of Drosophila show their banded structure well with ultra-violet light (Caspersson, 1936). In *Arbacia punctulata*, the chromatin network and chromosomes cannot be seen with visible light in the living egg, but show very nicely with ultra-violet light and appear exactly as they do in fixed and stained preparations. Judging from the absorption of ultra-violet light, compounds which absorb 2537 A° strongly are present in the Arbacia egg in the nucleolus, the chromatin network and the chromosomes, but not in the spindle fibers and asters, and not in the nuclear sap.

Cytoplasm of the Centrifuged Egg

When the *Arbacia punctulata* egg is centrifuged, the stratification is as follows: Oil at the centripetal pole, clear layer, a band of mitochondria, a large layer of yolk granules, and red pigment granules at the centrifugal pole; the nucleus lies in the clear layer under the oil cap (Harvey, 1932; 1940). In the living egg, the stratification is very striking (Photograph 10). The different materials stain selectively

with many vital dyes; e.g. the mitochondria stain purple with methyl green, the yolk and pigment stain blue with methylene blue (Harvey, 1941). When the centrifuged eggs are fixed, sectioned and stained with Heidenhain's haematoxylin, the layers are not so striking. After a Bouin fixation, the oil cap is not to be seen; it has apparently been dissolved by the fixative (Harvey, 1940, Photograph 124). However, with a formalin fixation the oil cap is preserved (Photographs 11, 12). The most striking difference between the living egg and the fixed and stained sections is in the clear layer. This is optically empty in the living egg, but is deeply stained in the haematoxylin preparations and is filled with very small granules (Photograph 12). In the formalin fixed sections, stained with haematoxylin, the different layers of mitochondria, yolk and pigment are scarcely distinguishable; they are apparent, however, in Bouin-fixed sections, especially when the haematoxylin preparations are counterstained (Harvey, 1940, Photograph 124).

It was found difficult to obtain good ultra-violet photographs of the living centrifuged egg, as it tended to burst with the pressure of the coverslip necessary to obtain the desired thinness for the light to penetrate. We therefore used unstained sectioned material; the Bouin fixative was not suitable for the ultra-violet, but the formalin fixation was quite satisfactory. The most absorbing area is the clear layer, which appears quite dark in the ultra-violet photographs, much darker than the nucleus (Photograph 13). A control, unstained, section photographed by visible light is shown in Photograph 11. The oil cap and the granular layers, mitochondria, yolk and pigment are somewhat but not markedly absorbing with ultra-violet light, and all about equally so. There is no special absorption by the mitochondria. The bulk of the protoplasmic nucleic acid compounds is therefore in the clear layer. This material is the ground substance or matrix of the uncentrifuged egg in which the granules lie. This is apparently also the important material for development, rather than the granules, since it has been shown that fractions of eggs, obtained by centrifugal force, may lack any one of the different types of granules and still develop (Harvey, 1932, 1936, 1940). It is of interest to find that it is the ground substance or matrix which contains the compounds which absorb in the nucleic acid region of the spectrum.

Summary

1. Photographs of living immature, mature and dividing eggs of *Arbacia punctulata*, taken with ultra-violet light of wave length 2537 A°, show an absorption, indicating presence of nucleic acid compounds, in the nucleolus, chromatin network and chromosomes. The photographs with ultra-violet light are similar to those of sections of fixed material stained with Heidenhain's haematoxylin taken with visible light. The chromatin network and chromosomes cannot be seen with visible light in the living egg.

2. In the centrifuged egg, with ultra-violet light, the clear layer shows greatest absorption, indicating the localization of nucleic acid compounds in this layer. With visible light, this layer appears dark and granular in sections stained with Heidenhain's haematoxylin, much as it does with ultra-violet light. It is optically empty in the living centrifuged egg with visible light. This layer represents the matrix or ground substance of the normal uncentrifuged egg. The matrix of the nucleus and the layers of granules (mitochondria, yolk, pigment and oil) are relatively non-absorbent with ultra-violet light.

LITERATURE CITED

BARBER, H. N., AND H. G. CALLAN, 1944. Distribution of nucleic acid in the cell. *Nature*, **153**: 109.

BĚLAŘ, K., 1929. Beiträge zur Kausalanalyse der Mitose. II. Untersuchungen an den Spermatocyten von Chorthippus (Stenobothrus) lineatus Panz. *Arch. f. Entw. mech.*, **118**: 359–484.

BLANCHARD, K. C., 1935. The nucleic acid of the eggs of Arbacia punctulata. *Jour. Biol. Chem.*, **108**: 251–256.

BRACHET, J., 1933. Recherches sur la synthèse de l'acide thymonucléique pendant le développement de l'oeuf d'Oursin. *Arch. de Biol.*, **44**: 519–576.

BRACHET, J., 1937. Remarques sur la formation de l'acide thymonucléique pendant le développement des oeufs à synthèse partielle. *Arch. de Biol.*, **48**: 529–548.

CALLAN, H. G., 1943. Distribution of nucleic acid in the cell. *Nature*, **152**: 503.

CASPERSSON, T., 1936. Über den chemischen Aufbau der Strukturen des Zellkernes. *Skand. Arch. f. Physiol.*, **73**: 1–151 (Suppl. 8).

CASPERSSON, T., AND SCHULTZ, 1940. Ribonucleic acids in both nucleus and cytoplasm, and the function of the nucleolus. *Proc. Nat. Acad. Sci.*, **26**: 507–523.

CHAMBERS, R., 1914. Some physical properties of the cell nucleus. *Science*, **40**: 824–827.

HARVEY, E. B., 1932. The development of half and quarter eggs of Arbacia punctulata and of strongly centrifuged whole eggs. *Biol. Bull.*, **62**: 155–167.

HARVEY, E. B., 1936. Parthenogenetic merogony or cleavage without nuclei in Arbacia punctulata. *Biol. Bull.*, **71**: 101–121.

HARVEY, E. B., 1940. A comparison of the development of nucleate and non-nucleate eggs of Arbacia punctulata. *Biol. Bull.*, **79**: 166–187.

HARVEY, E. B., 1941. Vital staining of the centrifuged Arbacia punctulata egg. *Biol. Bull.*, **81**: 114–118.

KÖHLER, A., 1904. Mikrophotographische Untersuchungen mit ultraviolettem Licht. *Zeit. f. wiss. Mikros.*, **21**: 129–165, 273–304.

LAVIN, G. I., 1943. Simplified ultraviolet microscopy. *Rev. Sci. Instr.*, **14**: 375–376.

LUCAS, F. F., AND M. B. STARK, 1931. A study of living sperm cells of certain grasshoppers by means of the ultra-violet microscope. *Jour. Morph.*, **52**: 91–107.

STEDMAN, E., AND E. STEDMAN, 1943a. Chromosomin, a protein constituent of chromosomes. *Nature*, **152**: 267–269.

STEDMAN, E., AND E. STEDMAN, 1943b. Distribution of nuclei acid in the cell. Reply to H. G. Callan. *Nature*, **152**: 503–504.

STEDMAN, E., AND E. STEDMAN, 1943c. Probable function of histone as a regulator of mitosis. *Nature*, **152**: 556–557.

WYCKOFF, R. W. G., AND A. H. EBELING, 1933. Some ultraviolet photomicragraphs made with different wave lengths. *Jour. Morph.*, **55**: 131–135.

XANTHOPHYLLS AND CAROTENES OF DIATOMS, BROWN ALGAE, DINOFLAGELLATES, AND SEA-ANEMONES

HAROLD H. STRAIN, WINSTON M. MANNING AND GARRETT HARDIN

(Carnegie Institution of Washington, Division of Plant Biology, Stanford University, California)

Diatoms, dinoflagellates and brown algae, the principal autotrophic organisms of the sea, contain chlorophyll *a*, chlorophyll *c* (Strain and Manning, 1942a; Strain et al, 1943) and complex mixtures of yellow pigments. These yellow carotenoid pigments, which appear to play a role in photosynthesis (Dutton and Manning, 1941), have now been investigated by means of the highly selective chromatographic adsorption method. They have been compared with similar xanthophylls and carotenes from other sources such as leaves, flowers, and sea-anemones.

In the past seventy-five years there have been many investigations of the carotenoid pigments of diatoms and of brown algae. β-Carotene is the principal carotene and fucoxanthin is the principal xanthophyll (Palmer, 1922; Kylin, 1927; Walker, 1935; Heilbron, 1942). One or two additional fucoxanthin-like xanthophylls (Kylin, 1927; Montfort, 1940; Seybold and Egle, 1938; Seybold et al, 1941; Handke, 1941) have been regarded as native pigments (Kylin, 1927), as oxidation products of the principal fucoxanthin (Heilbron and Phipers, 1935; Kylin, 1939) or as interconvertible fucoxanthin isomers (Strain and Manning, 1942b). Other xanthophylls reported in brown algae are: fucoxanthophyll (Tswett, 1906a), xanthophyll or lutein (Palmer, 1922; Kylin, 1927; Heilbron, 1942; Montfort, 1940; Seybold and Egle, 1938; Seybold et al, 1941; Handke, 1941; Heilbron and Phipers, 1935; Kylin, 1939; Willstätter and Page, 1914; Carter et al, 1939; Pace, 1941), phyllorhodin or zeaxanthin (Kylin, 1939), cryptoxanthin and isolutein (Pace, 1941). Phylloxanthin, first reported both in brown algae and in leaves of higher plants, was found to be similar to the violaxanthin of pansy flowers (Kylin, 1927; 1939; Heilbron, Parry and Phipers, 1935). Zeaxanthin has also been regarded as a post-mortem product of brown algae (Heilbron and Phipers, 1935). Additional xanthophylls of diatoms have been reported as xanthophyll or lutein (Carter et al, 1939; Palmer, 1922; Kylin, 1927; Heilbron, 1942; Montfort, 1940; Seybold and Egle, 1938; Seybold et al, 1941; Handke, 1941; Pace, 1941), zeaxanthin, cryptoxanthin and isolutein (Pace, 1941).

There have been few investigations of the xanthophylls of dinoflagellates. The principal xanthophyll, called *peridinin* (Schütt, 1890; Kylin, 1927; Seybold et al, 1941), resembles the pigment sulcatoxanthin subsequently isolated from sea-anemones (Heilbron, Jackson and Jones, 1935). Another pigment of dinoflagellates is similar to the strongly adsorbed xanthophylls prepared from higher plants (Kylin, 1927; Strain, 1938a). The presence of lutein (xanthophyll) has also been reported (Seybold et al, 1941).

Experimental

Plant material. Brown algae were collected on the rocky reefs near Half Moon Bay, California. They were: *Fucus furcatus, Hesperophycus Harveyanus, Pelvetiopsis limitata, Cystoseira Osmundacea, Laminaria Andersonii, Pterygophera californica, Egregia Menziesii, Macrocystis integrifolia, Nereocystis pyrifera.*

Two colonial diatoms collected in quantity at the seashore were *Navicula torquatum* and *Isthmia nervosa.* Other diatoms, grown in pure culture or in unialgal culture, included *Nitzschia closterium, Nitzschia palea, Stephanopyxis turris* and *Thalassiosira gravida* (Strain and Manning, 1942a; Strain et al, 1943).

A fresh-water dinoflagellate, *Peridinium cinctum* (Strain et al, 1943), and a fresh-water yellow-green alga, *Tribonema bombycinum,* were found growing in a high state of purity as natural "blooms." A small, unicellular, brown colored alga was obtained in great concentration from the tentacles of the large, common, Pacific Coast sea-anemone *Bunodactis (Cribrina) xanthogrammica* (Strain et al, 1943). The amounts of algal material employed for preparation of the xanthophylls varied from 1 or 2 gm. of the centrifuged unicellular algae to 15 or 20 gm. of the brown algae. Only fresh, living algae were utilized.

Extraction of pigments. Absolute methanol containing about 0.5 per cent dimethylaniline was usually employed to extract the pigments from algae (Strain, 1938b; Strain and Manning, 1942a; Strain et al, 1943). Alteration of the chlorophyll to products that were difficult to remove from the xanthophylls was retarded by extraction of the plant material at room temperature with such a large quantity of methanol that the total amount of water present was not over 2 to 5 per cent.

Chromatographic behavior of pigments. Separation of the xanthophylls from the chlorophylls in extracts of many algae through use of saponification methods was not feasible, because some of the xanthophylls were altered by alkali. Partition of the pigments between methanol and petroleum ether failed to remove alcohol-soluble chlorophyll *c* from the xanthophylls. Modifications of the chromatographic adsorption method were finally utilized for separation of the chlorophylls and carotenes from the xanthophylls and for further resolution of the xanthophylls and carotenes.

No single adsorption procedure sufficed to separate all the algal pigments from one another. Many of the pigments were decomposed slowly by adsorption upon magnesium oxide. After partial separation of the pigments by adsorption upon columns of sugar, the incompletely separated, weakly adsorbed xanthophylls could be resolved further by adsorption upon columns of magnesia or by readsorption upon columns of sugar using mixtures of various solvents for development of the chromatograms. Under the different conditions, the relative positions occupied by the xanthophylls on the columns were often quite different (Strain, 1942a; 1942b; LeRosen, 1942).

Chromatographic preparation of pigments. Pigments contained in the methanol extracts of algae were transferred to petroleum ether by the addition of this solvent and aqueous salt solution. The green petroleum ether solutions were washed with water to remove residual methanol, concentrated to about 40 ml. at a temperature never above 20°, and then filtered through adsorption columns of dry powdered sugar (Strain, 1942a; 1942b; Strain et al, 1943), usually 3 or 4 cm. by 20 to 27 cm. In order to carry the last portions of the weakly adsorbed carotenes

below the other pigments, the columns were then washed with a little fresh petroleum ether, followed by petroleum ether containing 0.5 per cent *n*-propanol and 0.5 per cent dimethylaniline.

For spectroscopic examination of the carotene mixture small portions of the percolates were evaporated to dryness at reduced pressure. The residual carotenes were dissolved in 95 per cent ethanol, and the absorption spectra of the solutions were determined. For further identification of the carotenes, the bulk of the petroleum ether percolates were extracted several times with very dilute hydrochloric acid (0.5 per cent) which removed the dimethylaniline. The carotenes were then identified by adsorption upon a column prepared from a mixture of Micron Brand magnesium oxide No. 2641 and heat-treated diatomaceous earth (Filter Aid 501) (Strain, 1938a; 1942b).

Continued washing of the sugar columns with petroleum ether containing 0.5 per cent *n*-propanol caused the weakly adsorbed xanthophylls to separate from the chlorophyll *a* and from the strongly adsorbed xanthophylls and chlorophyll *c*. Resolution of the strongly adsorbed xanthophylls proceeded much more rapidly when petroleum ether with about 2 per cent *n*-propanol was subsequently used to develop the chromatogram. In order to accelerate the separation of the pigments and to avoid contamination of the resolved compounds with isomerization products that formed slowly, the weakly adsorbed and the strongly adsorbed xanthophylls were usually prepared rapidly on separate columns. For similar reasons, only freshly prepared extracts of the algae were utilized.

Petroleum ether solutions of several different alcohols were tested as solvents for the separation of algal pigments adsorbed upon columns of sugar. The resolving effect of aliphatic alcohols of various molecular weights was roughly equivalent to the amount of hydroxyl group that was present in the petroleum ether solution. Alcohols of higher molecular weight, such as amyl alcohol, produced slightly better separations of the pigments than alcohols of low molecular weight. At concentrations above 3 per cent, methanol tended to separate from the petroleum ether as a distinct phase in the adsorption columns. Alcohols of high molecular weight remained in solution, but they were difficult to remove from the petroleum ether by extraction with water and thus hindered further purification of the pigments by readsorption.

Pigments separated upon the adsorption columns were often contaminated by trailing portions of the substances which had preceded them (Strain, 1942a). All pigment preparations were purified by readsorption under various conditions until superposable spectral curves were obtained. The small quantities of the readsorbed pigments and the lability of many of them precluded the use of purification procedures involving crystallization.

Determination of properties of xanthophylls and carotenes. The most useful properties proved to be *partition between immiscible solvents* such as aqueous methanol and petroleum ether, *color reactions* produced by strong acids such as concentrated hydrochloric acid in the presence of ether, *comparative adsorbabilities* usually determined by adsorption upon Tswett columns of a mixture by the substances to be compared (Strain, 1942b), and *spectral absorption characteristics.*

Spectral absorption properties of the xanthophylls, determined with a photoelectric spectrophotometer (Smith, 1936), are plotted as the so-called characteristic absorption curves, the plot of log log (I_0/I) *versus* wave-length. Because of the

Color of adsorbed pigments	Name of pigments	Wave-lengths of absorption maxima (ethanol, mμ)	
Light green	Chlorophyll *c*		
Orange	Neofucoxanthin A	447	
Yellow-orange	Neofucoxanthin B	446	
Orange	Fucoxanthin	453	
Yellow	Diadinoxanthin	448	478
Yellow	Diatoxanthin	453	481
Green	Chlorophyll *a*		
Traces of yellow,	Xanthophylls		
	Chlorophyll *a'*		
green and gray	Pheophytin *a*		
Yellow	Carotenes		

FIGURE 1. Diatom pigments separated by adsorption upon a column of powdered sugar.

lability of the pigments, the small quantities obtained, and the presence of colorless concomitants, determination of the specific absorption coefficients was not feasible. Relative absorption values were always determined within a few hours after purification of the pigments by adsorption.

Results

Xanthophylls of diatoms. All the diatoms yielded the same series of xanthophyll pigments shown in Figure 1. However, different species showed significant variations in the relative amounts of the pigments.

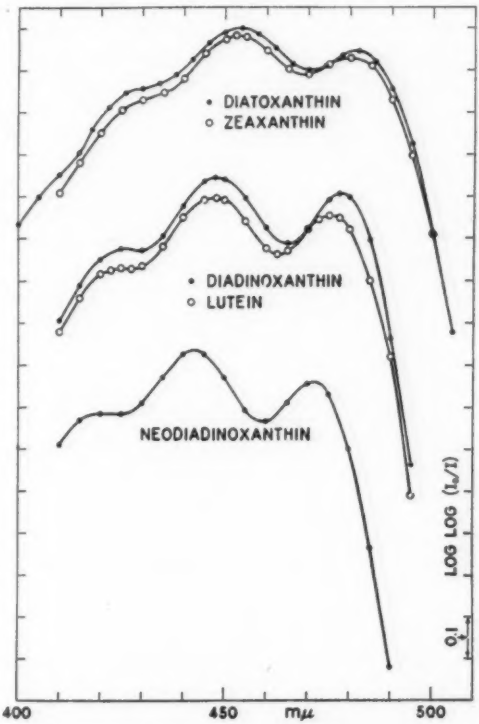

Figure 2. Characteristic spectral absorption curves of diatoxanthin, zeaxanthin, diadinoxanthin, luetin and neodiadinoxanthin. Solvent ethanol (95 per cent).

Diatoxanthin, a new xanthophyll, formed a paler but distinctly more orange band than the pigment adsorbed above it. Purified by readsorption, diatoxanthin yielded the spectral absorption curve shown with the similar zeaxanthin curve (Strain, 1938a) in Figure 2.

When dissolved in ether and treated with concentrated hydrochloric acid, diatoxanthin did not yield colored products. A mixture of diatoxanthin and zeaxan-

thin (the latter from the calyx of *Physalis alkekengi*) (Strain, 1938a) adsorbed from petroleum ether on a column of sugar and washed with petroleum ether containing one per cent methanol was resolved very slowly, zeaxanthin forming the lower band.

Diatoxanthin, dissolved in *n*-propanol and heated on a boiling water bath for one hour, underwent reversible isomerization, yielding some *neodiatoxanthin*. This second pigment was more strongly adsorbed and formed a yellower band, on a column of sugar, than the unchanged diatoxanthin (petroleum ether with 0.5 per cent *n*-propanol as wash liquid). Spectral absorption maxima of neodiatoxanthin were less pronounced and occurred at wave-lengths about 6 mμ shorter than those of diatoxanthin. By analogy with isomerization reactions of other carotenoid pigments (Strain, 1941; Polgár and Zechmeister, 1942), neodiatoxanthin probably represents a labile modification of the polyene system present in the molecule, diatoxanthin representing the stable or *trans* configuration.

In respect to color and position on the sugar columns, adsorbed neodiatoxanthin resembled the yellow band containing diadinoxanthin found next above the diatoxanthin when pigments of diatoms were adsorbed (see Fig. 1). However, diadinoxanthin and neodiatoxanthin did not yield identical spectral absorption curves. When a mixture of the two pigments was adsorbed on a column of sugar (petroleum ether plus 0.5 per cent *n*-propanol as wash liquid), neodiatoxanthin was adsorbed above diadinoxanthin.

Except for slight interconversion, neither diatoxanthin nor neodiatoxanthin was altered by a strong solution of potassium hydroxide in methanol. The behavior of these two pigments upon heating their solutions indicates that neither one is a labile isomer of zeaxanthin. Not more than traces of neodiatoxanthin or of zeaxanthin could have been present in the diatoms.

Diadinoxanthin (see Fig. 1) resembled lutein in respect to its spectral absorption (see Fig. 2) and color reactions. Readsorbed with lutein upon a column of sugar (petroleum ether with 0.5 per cent *n*-propanol as wash liquid), the diadinoxanthin moved through the column only about two-thirds as fast as the lutein.

As illustrated in Figure 1, the leading portion of the band of adsorbed diadinoxanthin became quite diffuse while the upper boundary of the band remained sharp and concentrated. This unique distribution of the pigment adsorbed on the column was observed after the diadinoxanthin had been purified in various ways. Spectroscopic properties of the pigment from the leading and trailing portions of the band were identical. Readsorption of each of these fractions upon fresh columns of sugar again yielded bands with diffuse leading portions.

In solution in hot *n*-propanol, diadinoxanthin underwent rapid, reversible isomerization yielding the similar, more adsorbed *neodiadinoxanthin*. Spectral absorption properties of the two pigments may be compared in Figure 2.

Fucoxanthin and its isomers (Strain and Manning, 1942b) contained in the extracts of diatoms moved slowly through the adsorption columns as indicated in Figure 1. Instead of the names suggested previously for the isomeric fucoxanthins (Kylin, 1927; 1939; Strain and Manning, 1942b), it is now proposed to call the principal isomer *fucoxanthin*, the other isomers *neofucoxanthin* A and *neofucoxanthin* B in conformity with the nomenclature of isomeric carotenoid pigments introduced by Zechmeister and his co-workers (Polgár and Zechmeister, 1942; and incl. refs.).

When the pigments were extracted quickly from diatoms and adsorbed on sugar from solution in petroleum ether containing 2 per cent *n*-propanol, the same mixture of pigments was always obtained. Adsorption of the principal fucoxanthin isomer under these same conditions yielded only traces of the other two compounds. This indicated that all three fucoxanthins may be normal constituents of the diatoms.

Interconversion of the three fucoxanthins proceeded rapidly in ethanol at 76° and slowly at 20°. Interconversion was accelerated by light and by substances upon which the pigments were strongly adsorbed, as for example by powdered glass or sugar when petroleum ether was used as solvent. This interconversion was also catalysed by iodine, but when bases such as pyridine and dimethylaniline were not added to neutralize traces of hydriodic acid that were formed (Strain, 1941), other pigments were produced. About 90 per cent of the equilibrium mixture, obtained by heating a solution of any of the isomers, was the stable fucoxanthin.

When dissolved in ether and treated with concentrated hydrochloric acid, each of the fucoxanthin isomers was converted into acid-soluble blue compounds. Treated with alcoholic potassium hydroxide the isomers formed pale yellow pigments that gave a blue color reaction with very dilute hydrochloric acid. The reaction with alkali proceeded in vacuum and in hydrogen as well as in air.

In order to obtain consistent spectral absorption curves for the isomeric fucoxanthins it was necessary to develop the chromatograms directly with petroleum ether containing 2 per cent *n*-propanol and 0.5 per cent dimethylaniline and to *work rapidly*. With these precautions, the same spectral curves were obtained whether the pigments were prepared from the diatoms or by interconversion from any of the other isomers (see Fig. 3). Noteworthy is the presence of only one definite spectral absorption maximum in the curve of each pigment. The spectral absorption curve of a petroleum ether solution of the principal fucoxanthin isomer, which is also shown in Figure 3, exhibits very distinct maxima, in sharp contrast to the curve for an ethanol solution. These spectral properties correspond to those of other carotenoid pigments that contain carbonyl groups in conjugation with the polyene structure in the molecule (Heilbron and Lythgoe, 1936).

These results on the xanthophylls of diatoms are contrary to the numerous reports (*loc. cit.*) that lutein is a constituent of these organisms. It is probable that diadinoxanthin may have been mistaken for lutein, and diatoxanthin may have been mistaken for zeaxanthin.

Carotenes of diatoms. Five of the six diatom species investigated contained principally β-carotene with only traces of other more adsorbed polyene hydrocarbons. One species, *Navicula torquatum*, contained the weakly adsorbed ϵ-carotene in addition to β-carotene (Strain and Manning, 1943a).

Effect of light of various spectral qualities on the formation of xanthophylls in diatoms. Pure cultures of *Nitzschia closterium* which were grown continuously in light from fluorescent "snow white" tubular lamps exhibited a constant ratio between the several xanthophyll pigments. When these cultures were placed in the red light from neon tubular lamps there was a gradual increase in the proportion of diadinoxanthin. After 20 days, when the culture had increased manyfold, the proportion of diadinoxanthin to other pigments was nearly twice as great as that in the cells grown in light from white fluorescent lamps. At this time the proportions between the other xanthophylls had changed very little.

These experiments demonstrated that the proportions of the carotenoid pigments of this photosynthetically active organism vary in response to changes in the environment. Whether this change results from variation in the rate of synthesis or degradation of the different pigments or whether it results from accelerated growth of a strain of the organism that contains different proportions of the xanthophylls is not yet known.

Xanthophylls of brown algae. Adsorption of the pigments of brown algae upon columns of sugar yielded a series of bands similar to those obtained from diatoms except that there was much less yellow pigment between the fucoxanthin and the chlorophyll *a*. Traces of two pigments, adsorbed as pale, narrow bands above

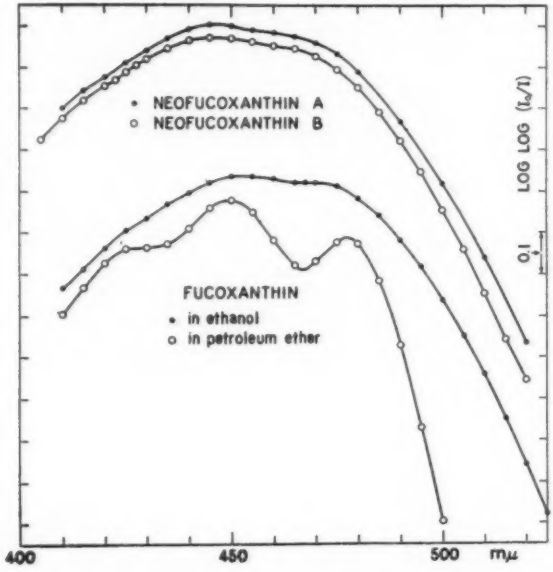

FIGURE 3. Characteristic spectral absorption curves of neofucoxanthin A, neofucoxanthin B and fucoxanthin dissolved in ethanol (95 per cent) and of fucoxanthin dissolved in petroleum ether.

chlorophyll *a*, appeared to be the diatoxanthin and the diadinoxanthin separated from the extracts of diatoms. The relative amounts of these two xanthophylls varied in different extracts of the same organism and in different species of brown algae. In view of the very small quantities of these two xanthophylls and their apparent absence in many species, it is uncertain whether the pigments came from the brown algae themselves or from diatoms that might have contaminated them.

When the pigments from brown algae were first adsorbed, a distinct yellow band usually appeared just below the orange fucoxanthin band. As the column was developed with petroleum ether containing propanol, this yellow band was often over-run by the orange fucoxanthin band. Under these conditions, the yellow pig-

ment, which exhibited spectral properties similar to those of violaxanthin, was observed again between the fucoxanthin and the neofucoxanthin B. If, however, the adsorbed pigments were washed with petroleum ether containing about 5 per cent acetone, the yellow band continued to advance ahead of the fucoxanthin, occasionally separating into two contiguous yellow bands. Pigment from the lower of these two yellow bands resembled the violaxanthin of leaves (Strain, 1938a), as is shown by the spectral absorption curves in Figure 4. Pigment from the upper yellow band exhibited a spectral absorption curve which (when corrected for absorption due to remaining violaxanthin) resembled the spectral curve of the flavoxanthin of leaves

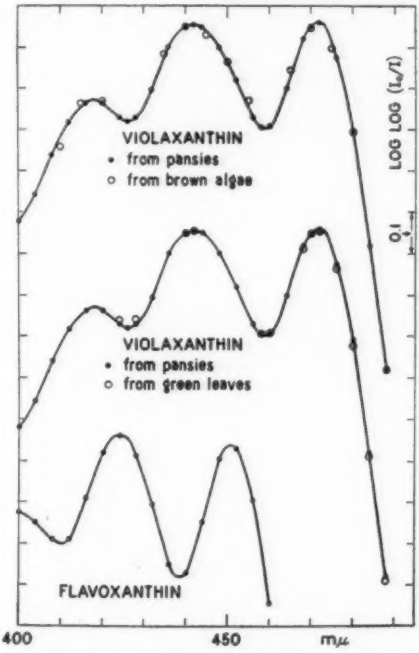

FIGURE 4. Characteristic spectral absorption curves of flavoxanthin and of violaxanthin from pansies, leaves and brown algae. Solvent, ethanol (95 per cent).

(Strain, 1938a). This flavoxanthin-like xanthophyll occurred in such small quantities that it could not be compared in other respects with the similar pigment of leaves.

The violaxanthin-like pigment from brown algae was found to be spectroscopically identical with violaxanthin prepared from leaves and from pansy flowers as described below (Fig. 4). Violaxanthin preparations from the several sources were also chromatographically identical. Mixtures of these preparations were inseparable by adsorption upon columns of magnesia (petroleum ether with 25 per cent acetone as wash liquid) or by adsorption upon columns of sugar (petroleum ether with 0.5 per cent *n*-propanol as wash liquid).

Changes in the relative positions of violaxanthin and of fucoxanthin, first observed when extracts of brown algae were adsorbed upon columns of sugar with different solvents as wash liquids, have been obtained with highly purified preparations of these two xanthophylls. With petroleum ether or with petroleum ether containing 5 per cent acetone as solvent, violaxanthin was adsorbed below fucoxanthin. With petroleum ether containing 0.5 per cent *n*-propanol as solvent, violaxanthin was adsorbed above fucoxanthin. Separated below fucoxanthin by adsorption from solution in petroleum ether containing acetone, violaxanthin gradually became adsorbed above the fucoxanthin when the column was washed with petroleum ether containing *n*-propanol.

Fucoxanthin and its isomers separated from the brown algae were identical with those separated from the diatoms as indicated by identical spectral absorption curves, adsorbability, and color reactions. Pigments that were extracted rapidly from fresh algal material and adsorbed from petroleum ether containing 2 per cent *n*-propanol yielded all three isomers; hence, these three pigments probably represent normal constituents of brown algae as well as of diatoms.

When the pigments of brown algae were adsorbed from solution in petroleum ether containing 2 per cent *n*-propanol, traces of xanthophylls with absorption spectra similar to that of neoxanthin from leaves (Strain, 1938a) were observed below and just above the fucoxanthin. Because of their weak adsorbability, neither of these pigments could have been neoxanthin. Their relations to other xanthophylls of similar spectra (Fig. 6) were not established. Traces of a xanthophyll with spectral properties similar to those of dinoxanthin (*vide infra*) were also observed on the column below the adsorbed fucoxanthin.

Carotenes separated from the brown algae were composed principally of the *beta* isomer with traces of more adsorbed carotenes. Neither α-carotene nor ε-carotene was observed.

These results on the carotenoid pigments of brown algae support the early observation of Tswett that fucoxanthophyll, the xanthophyll of these organisms, in addition to fucoxanthin, is different from xanthophyll (lutein) of leaves. They confirm the statement of Kylin (1927; 1939) that violaxanthin (phylloxanthin) is present in brown algae. They are in disagreement with the claims of Kylin and of many others, that lutein and zeaxanthin occur in many species of brown algae (some of which belong to the same genera as several of the species examined by us). In certain species of Laminaria and of Fucus, Heilbron and co-workers (Heilbron, 1942; Heilbron and Phipers, 1935; Carter et al, 1939) could not detect any xanthophylls other than fucoxanthin.

Our observations both on diatoms and on brown algae support Kylin's original statement (1927) and other recent claims (Montfort, 1940; Seybold and Egle, 1938; Strain and Manning, 1942b) that the several fucoxanthins are normal constituents of the plant cells. They are at variance with Heilbron and Phipers' contention that the additional fucoxanthin they observed is an oxidation product of the principal fucoxanthin. All these conclusions are difficult to reconcile with Kylin's latest statement (1939) that the pigment he regarded as fucoxanthin B may also have been an oxidation product. Zeaxanthin, reported as a normal constituent of brown algae by Kylin and by Pace and as a post-mortem product by Heilbron and Phipers, was not detected in our experiments.

Kylin (1939) has asserted that the violaxanthin of brown algae and of leaves

Color of adsorbed pigments	Name of pigments	Wave-lengths of absorption maxima (ethanol, mμ)	
Light green	Chlorophyll *c*		
Red-orange	Neoperidinin	464	
Red-orange	Peridinin	475	
Yellow	Neodinoxanthin	438	466
	Neodiadinoxanthin	442	471
Yellow	Dinoxanthin	441.5	471
	Diadinoxanthin	448	478
Green	Chlorophyll *a*		
Traces of yellow, green and gray	Xanthophylls		
	Chlorophyll *a'*		
	Pheophytin *a*		
Yellow	Carotenes		

FIGURE 5. Pigments of *Peridinium cinctum* (and of an alga from a sea-anemone) separated by adsorption upon a column of powdered sugar.

is identical with Tswett's (1906b) β-xanthophyll, the only carotenoid observed above chlorophyll *b* when leaf pigments were adsorbed upon columns of precipitated chalk with carbon disulfide as wash liquid. Repetition of Tswett's experiment using extracts of sunflower leaves has revealed that neoxanthin (Strain, 1938a) with traces of other xanthophylls forms the topmost yellow band. On columns of sugar with petroleum ether containing 0.5 per cent *n*-propanol as wash liquid, neoxanthin also formed the topmost band. Only after prolonged development of the chromatogram did violaxanthin appear above the band of adsorbed chlorophyll *b*. Under these conditions traces of a flavoxanthin-like xanthophyll appeared between the neoxanthin and the violaxanthin. This indicates that Tswett's β-xanthophyll may have been a mixture containing principally neoxanthin.

Xanthophylls of a dinoflagellate. Pigments extracted from the dinoflagellate *Peridinium cinctum* and adsorbed on a column of sugar yielded the series of bands shown in Figure 5. The yellow band which occurred next above the chlorophyll *a* proved to be a mixture of at least two pigments which were separated from each other by readsorption upon a column of sugar (petroleum ether with 5 per cent acetone or 0.5 per cent *n*-propanol as wash liquid). Diadinoxanthin, which formed the lower band, was spectroscopically and chromatographically identical with this pigment prepared from diatoms.

Dinoxanthin, separated from the diadinoxanthin by readsorption of the mixture from the original column, exhibited spectral absorption properties similar to those of violaxanthin and of taraxanthin (Fig. 6). In spite of the similarity in their spectra, these three pigments are distinct substances as demonstrated by their different adsorbabilities and their color reactions with acid. When the pigments were adsorped on sugar from solution in petroleum ether containing one per cent *n*-propanol, the adsorption order was: violaxanthin (topmost), dinoxanthin, and taraxanthin. On magnesia with petroleum ether containing 25 per cent acetone as solvent, the adsorption order was: dinoxanthin, violaxanthin and taraxanthin. Dissolved in ether and treated with concentrated hydrochloric acid, dinoxanthin and taraxanthin yielded only a trace of blue color in the acid layer whereas violaxanthin yielded a stable, deep blue color in the acid. Prior treatment of dinoxanthin with alcoholic potassium hydroxide did not affect its reaction with concentrated hydrochloric acid. None of these pigments was interconvertible by the action of heat on its solution.

There were indications that traces of a flavoxanthin-like xanthophyll occurred in admixture with the diadinoxanthin and the dinoxanthin. The quantities present were insufficient to permit definite identification.

As illustrated in Figure 5, there was always a small yellow band just below the principal red-orange band obtained by adsorption of the pigments of the dinoflagellates. Readsorption of the pigment from this band on a fresh column yielded neodiadinoxanthin as the lower band and an isomer of dinoxanthin as the upper band. In Figure 6, a spectral absorption curve of this second pigment, *neodinoxanthin,* may be compared with the curve of the more stable interconvertible dinoxanthin. Color and solubility reactions of the two pigments were similar.

In all the experiments, even though rapid and mild conditions of extraction and adsorption were employed, both neodinoxanthin and neodiadinoxanthin were observed in the extracts of the dinoflagellates. Because of the ease and rapidity with

which these two isomers are formed, it is not certain that they are normal constituents of *Peridinium*.

Peridinin, obtained from the principal red-orange band (illustrated in Fig. 5), exhibited some noteworthy properties. So far as can be ascertained, this pigment

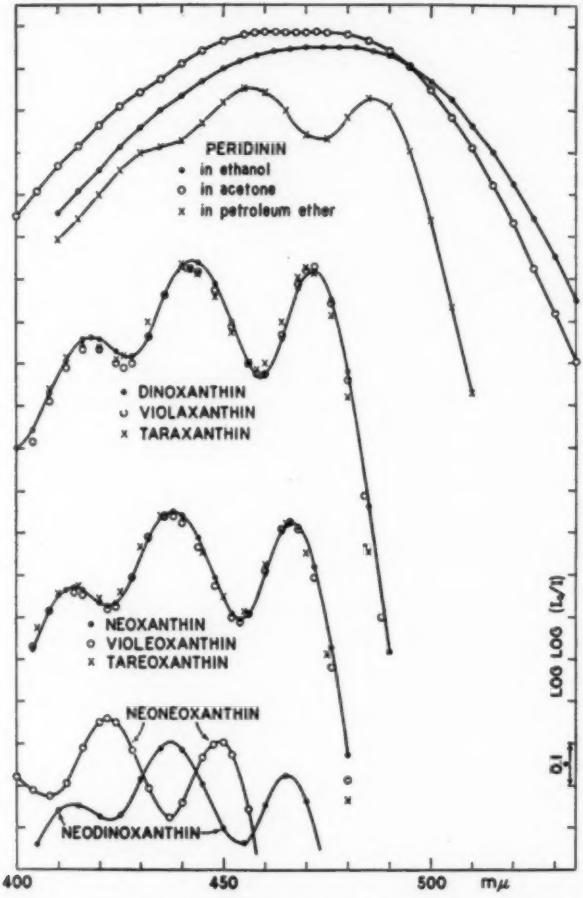

FIGURE 6. Characteristic spectral absorption curves of peridinin, dissolved in various solvents, and of dinoxanthin, violaxanthin, taraxanthin, neoxanthin, violeoxanthin, tareoxanthin, neodinoxanthin and neoneoxanthin dissolved in ethanol (95 per cent).

is the one observed in several species of *Peridinium* by Schütt (1890), by Kylin (1927), and by Seybold, Egle and Hülsbruch (1941). In solubility, peridinin resembled fucoxanthin, being extremely soluble in alcohols and but slightly soluble in petroleum ether. On sugar, it was much more adsorbed than fucoxanthin from

which it could be separated readily by development of the chromatogram with petroleum ether containing 2 or 3 per cent *n*-propanol. Peridinin was decomposed by alkalies, yielding paler pigments that did not give blue products when dissolved in ether and treated with concentrated hydrochloric acid. Peridinin itself, in contrast to fucoxanthin, did not yield a blue color with concentrated hydrochloric acid. This behavior of peridinin, like that of diadinoxanthin, is an exception to the rule that strongly adsorbed, alcohol-soluble xanthophylls yield a blue color with concentrated hydrochloric acid.

When dissolved in chloroform and treated with a solution of antimony trichloride in chloroform, peridinin formed a purple-orange solution that faded slowly to a lighter orange. Dissolved in chloroform and treated with concentrated sulfuric acid, peridinin caused the acid layer to turn deep blue. Crystals of peridinin, which formed readily when the solutions were concentrated, were turned deep blue by concentrated sulfuric acid.

Dissolved in methanol or in ethanol, peridinin exhibited strong absorption of light ranging from the violet to the yellow region of the spectrum. The spectral curve of alcohol and of acetone solutions showed a single broad absorption maximum. On the other hand, a solution of peridinin in petroleum ether exhibited two pronounced absorption maxima (see Fig. 6). Addition of as little as 2.5 per cent ethanol to a petroleum ether solution of peridinin caused a marked broadening of the absorption maxima. The petroleum ether solutions were quite yellow whereas the alcohol solutions were distinctly orange-red. By analogy with spectra of other carotenoid pigments (Heilbron and Lythgoe, 1936), these spectral properties indicate that the peridinin molecule contains at least one carbonyl group in conjugation with the polyene system.

Solutions of peridinin in carbon disulfide were pink-orange to red-orange in color. The peridinin in these solutions was very strongly adsorbed on sugar yielding a red adsorbate.

A dilute, yellow solution of peridinin in petroleum ether turned brick red when shaken with distilled water, and the pigment collected at the interface. This effect is similar to the color change observed when the peridinin is adsorbed on solids. Upon adsorption on solids or at a liquid-liquid interface, fucoxanthin also behaved similarly, but neoxanthin, which was more adsorbed than peridinin or fucoxanthin (see below), retained its characteristic yellow shade.

Peridinin, like other more typical xanthophylls, was moderately resistant to oxidation by atmospheric oxygen. Solutions of this xanthophyll in ethanol or in petroleum ether stored in open vessels in the dark were not bleached perceptibly in 2 or 3 months. In diffuse light from north windows, the solutions became colorless in a few weeks. In direct sunlight the solutions were decolorized in a few days.

When solutions of peridinin in *n*-propanol were heated on a boiling water bath, the pigment was isomerized rapidly. Products obtained by heating the solution for 2 hours were transferred to petroleum ether and adsorbed on a column of sugar using petroleum ether with 3 per cent *n*-propanol for development of the chromatogram. This resulted in the separation of a pale, diffuse red-orange band containing *neoperidinin* above the band containing the unchanged peridinin. Faint traces of other red-orange bands were adsorbed above the neoperidinin and very small quantities of still another pigment were adsorbed below the peridinin. In ethanol, all these pigments exhibited spectral absorption curves similar to that of peridinin but

their absorption maxima occurred at shorter wave-lengths. Traces of all these pigments were often observed when extracts of Peridinium were adsorbed upon columns of powdered sugar.

The carotene mixture extracted from *Peridinium cinctum* was composed almost entirely of β-carotene. The quantity of carotene present in the fresh centrifuged cells was greater than that obtained from the diatoms.

Xanthophylls of an alga from a sea-anemone. Algae squeezed from the tentacles of the sea-anemone, *Bunodactis xanthogrammica,* yielded the same series of pigments obtained from the dinoflagellates and tabulated in Figure 5. These pigments are constituents of the alga, not of the anemone.

Color reactions already described for peridinin from Peridinium were identical with those described for sulcatoxanthin from the sea-anemone *Anemonia sulcata* (Heilbron, Jackson and Jones, 1935). The absorption curve of peridinin dissolved in carbon disulfide exhibited two well-defined absorption maxima at 483 and 516 mμ, in good agreement with the values of 482 and 516 mμ reported for sulcatoxanthin. On the basis of available evidence, it appears that sulcatoxanthin is none other than the xanthophyll peridinin. As we have not been able to obtain the anemone *Anemonia sulcata* for analysis of its pigments, it is impossible to say whether the peridinin originates in the tissue of that animal or in the cells of the algae that are known to inhabit it (Fulton, 1922).

The identity of the pigments obtained from the symbiotic alga of Bunodactis and from the dinoflagellate suggests that both organisms may belong to the same or to related plant groups. From a microscopical examination of the symbiotic alga, Dr. H. W. Graham of Mills College concluded that this organism is not a dinoflagellate. It has been suggested that "zooxanthellae," the symbiotic algae of many sea anemones, may belong to another class of organisms, the cryptomonads (Fulton, 1922). If this is true, the autotrophic, free-living cryptomonads may also contain the same pigments found in the dinoflagellates.

Pigments of a yellow-green alga. The xanthophylls of *Tribonema bombycinum* proved to be different from those of all the other algae that we have investigated. Neither lutein nor any other common xanthophyll was observed. The several xanthophylls that were present formed pale yellow bands on the sugar columns, but these pigments have not been definitely identified. The carotene consisted almost entirely of the *beta* isomer. Tribonema did not contain chlorophylls *b, c,* or *d.*

Xanthophylls of dandelion flowers. For comparison with dinoxanthin, taraxanthin was prepared from flowers of the dandelion *Taraxacum officinalis* (Kuhn and Lederer, 1931). To this end, about 10 gms. of fresh dandelion flowers from which the stems and sepals had been cut were extracted with absolute ethanol; the extract was treated with an excess of potassium hydroxide; and after completion of the saponification, the xanthophylls were transferred to petroleum ether and adsorbed upon a column (3.7 by 22 cm.) of magnesia and siliceous earth (1:1). The chromatogram was then developed with petroleum ether containing 25 per cent acetone. Taraxanthin proved to be the principal, least adsorbed xanthophyll. After purification by readsorption it yielded the spectral absorption values shown in Figure 6.

Above the taraxanthin there were two or three adjoining bands one of which contained lutein. Above this group of bands, there appeared a yellow band which

yielded a hitherto undescribed pigment. This new xanthophyll, for which we propose the name *tareoxanthin*, exhibited a spectral curve almost identical in shape with that of neoxanthin from leaves (see Fig. 6) (Strain, 1938a).

Just above the band containing the tareoxanthin on the column with the adsorbed dandelion pigments there appeared a lemon yellow band. Xanthophyll eluted from this band exhibited a spectral absorption curve almost identical with that reported from flavoxanthin in ethanol (Strain, 1938a). Unlike flavoxanthin from other sources (Strain, 1938a; Kuhn and Brockmann, 1932), this xanthophyll from dandelions did not yield a blue product when dissolved in ether and treated with concentrated hydrochloric acid. This fact suggests that the "flavoxanthin" isolated from dandelion flowers by Karrer and Rutschmann (1942a) may not have been identical with flavoxanthin from other plants.

The mixture of dandelion xanthophylls was also resolvable by adsorption upon columns of sugar when petroleum ether containing one per cent *n*-propanol was used as solvent. Under these conditions the taraxanthin was adsorbed above the lutein.

Xanthophylls of pansy flowers. Violaxanthin, for comparison with the xanthophylls of algae, was prepared from the yellow flowers of *Viola tricolor* (Kuhn and Winterstein, 1931). Xanthophyll esters in the extracts of the flowers were saponified, and the free xanthophylls were crystallized from petroleum ether and aqueous alcohol. After recrystallization from petroleum ether and aqueous alcohol, the violaxanthin was purified further by adsorption on columns of magnesia and of sugar.

When the xanthophylls in the mother liquors from the violaxanthin preparation were adsorbed on magnesia or on sugar, relatively large quantities of a strongly adsorbed xanthophyll were observed (Karrer and Rutschmann, 1942b). This pigment, for which the name *violeoxanthin* is proposed, exhibited a spectral absorption curve almost identical with those of neoxanthin and of tareoxanthin (Fig. 6).

In spite of the similarity between the spectral absorption curves of neoxanthin, tareoxanthin, and violeoxanthin, these three pigments were readily separable by adsorption. Upon columns of magnesia with petroleum ether containing 25 per cent acetone as solvent or upon columns of sugar with petroleum ether containing one per cent *n*-propanol as solvent, neoxanthin formed the uppermost band, violeoxanthin the middle band, and tareoxanthin the lowest band. Dissolved in ether and treated with concentrated hydrochloric acid, violeoxanthin yielded an intensely blue, acid-soluble product whereas neoxanthin yielded no color, and the tareoxanthin yielded only traces of blue in the acid.

Violaxanthin from leaves. Violaxanthin was rapidly preparable from leaves by adsorption of a petroleum ether solution of the extracted pigments upon a column of magnesia and siliceous earth followed by development of the chromatogram with petroleum ether containing 25 per cent anhydrous acetone. Under these conditions, the violaxanthin was adsorbed far below the chlorophylls and below lutein. As a consequence it was not contaminated with other leaf pigments and was nearly spectroscopically homogeneous after a single adsorption.

Neoxanthin from leaves. When the pigments extracted from leaves were dissolved in petroleum ether and adsorbed upon a column of heat-treated siliceous earth (Filter Aid 501), the chlorophylls and xanthophylls were more adsorbed than upon a column of sugar. As the adsorbed pigments were washed with petroleum

ether containing 0.5 per cent *n*-propanol, neoxanthin formed the uppermost yellow band. Owing to the high filtration rate of the columns, considerable neoxanthin could be separated in a few minutes. Purified by readsorption upon fresh columns, the neoxanthin exhibited a spectral absorption curve identical with that of material prepared by adsorption upon magnesia from solution in dichloroethane (Strain, 1938a). It did not exhibit a blue color when dissolved in ethyl ether and treated with concentrated hydrochloric acid.

Dissolved in *n*-propanol and heated on a boiling water bath for several hours, neoxanthin was converted into two isomers that were more adsorbed than the unchanged neoxanthin. The most adsorbed isomer, *neoneoxanthin* A, exhibited spectral absorption maxima at wave-lengths almost identical with those reported for the absorption maxima of flavoxanthin, but its absorption maxima were not so pronounced (Fig. 6). These isomerization experiments indicate that neoxanthin contains the stable form of the chromophoric polyene group.

Both violaxanthin and neoxanthin were obtained from leaves that had been extracted at room temperature, from those that had been killed by boiling and from those that had been extracted with alcohol containing much dimethylaniline. These xanthophylls were also obtained when the leaf extracts were adsorbed directly on magnesia, on sugar or on siliceous earth or when they were adsorbed after treatment with alcoholic potassium hydroxide. They were not formed from lutein when this xanthophyll was exposed to the conditions utilized for the extraction and separation of the leaf pigments. These observations provide further indication that neoxanthin and violaxanthin are normal constituents of green leaves.

Relative adsorbabilities of xanthophylls. In addition to those reversals in the relative positions of adsorbed xanthophylls already reported, dinoxanthin was found to be adsorbed above diadinoxanthin in columns of sugar (solvents: petroleum ether containing 0.5 per cent *n*-propanol, 5 per cent acetone or 8 per cent methylisobutylketone). But, on magnesia with acetone or methylisobutylketone as solvents, dinoxanthin was adsorbed below diadinoxanthin.

A mixture of lutein and zeaxanthin was not readily separable on a column of sugar when petroleum ether with 5 per cent acetone was used as solvent. Adsorbed upon magnesia from solution in petroleum ether containing 25 per cent acetone, zeaxanthin was adsorbed far above lutein.

TABLE I

Relative positions of xanthophylls on columns of magnesia and of sugar with various solvents

	Magnesia	Magnesia	Sugar	Sugar
Solvent.....	Dichloro-ethane	Petroleum ether + 25% acetone	Petroleum ether + 25% acetone	Petroleum ether + 1% *n*-propanol
Xanthophylls...	Neoxanthin Violaxanthin Zeaxanthin Lutein	[Neoxanthin * [Zeaxanthin Lutein Violaxanthin	Neoxanthin Violaxanthin [Zeaxanthin * [Lutein	Neoxanthin Violaxanthin [Zeaxanthin * [Lutein

* Brackets indicate pigments that did not separate into bands.

Relative positions of the xanthophylls in the columns have been found to vary with changes in either the solvent or the adsorbent. This is illustrated by the results summarized in Table I.

Because knowledge of the relative positions of known xanthophylls in the adsorption columns aids in the preparation and identification of these pigments from various sources, the adsorption orders of many of the xanthophylls described in this paper are listed in Table II. For these comparisons, mixtures of two or three of

TABLE II

Relative positions of xanthophylls on columns of magnesia and of sugar

	Magnesia	Sugar
Solvent	Petroleum ether + 25% Acetone	Petroleum ether + 0.5–1% *n*-propanol
Xanthophylls	Neoneoxanthin A	Neoxanthin
	Neoneoxanthin B	Peridinin
	[Neoxanthin *	Neofucoxanthin A
	[Zeaxanthin	Neofucoxanthin B
	Fucoxanthin	Violeoxanthin
	Peridinin	Violaxanthin
	[Violeoxanthin *	Fucoxanthin
	[Lutein	Tareoxanthin
	Dinoxanthin	Taraxanthin
	[Tareoxanthin *	[Zeaxanthin *
	[Violaxanthin	[Lutein
	Taraxanthin	Cryptoxanthin
	Cryptoxanthin	

* Brackets indicate pigments that did not separate into discrete bands.

the xanthophylls, dissolved in petroleum ether, were adsorbed, and the chromatograms were then developed with petroleum ether containing the polar solvent.

Changes in the relative adsorbabilities of the xanthophylls with variations of the solvent are apparently related to the solubilities of the pigments as well as to effects of the solvents upon the chromophoric groups (see Fig. 6). For example, peridinin and fucoxanthin, which are adsorbed below zeaxanthin when acetone is used as solvent, are more soluble in this ketone than is zeaxanthin.

DISCUSSION

Algal pigments. A number of new xanthophylls have been isolated from the algae investigated. Additional pigments might be obtained by the use of larger quantities of plant material followed by readsorption of the minor bands on smaller columns. Utilization of additional refinements in technique may result in further resolution of the pigments described in this paper, especially the isomerization products of the stable xanthophylls. However, it seems improbable that such pigments would represent a considerable proportion of the total xanthophylls.

The relatively great adsorbability of the algal xanthophylls indicates that these hydroxy compounds do not occur in the form of esters. In this respect, xanthophylls of algae resemble those of the chloroplasts of higher plants (Strain, 1938a).

Occurrence of the unesterified xanthophylls in the photosynthetic apparatus contrasts with the occurrence of esterified xanthophylls in the yellow chromoplasts of fruits and flowers.

Among the algae, the xanthophylls have been found to vary in three ways. In a given species, the proportions of the several pigments may alter in response to changes in the environment. In different plant classes, the xanthophylls differ in kind as well as in quantity. In some plant groups spatially unstable xanthophylls occur along with the stable or *trans* xanthophylls.

Of all the pigments now considered to comprise a part of the photosynthetic apparatus of autotrophic plants, the xanthophylls vary most. Except for a few specialized, pigmented bacteria, autotrophic plants contain chlorophyll *a* and β-carotene as the principal representatives of these two ubiquitous types of plant pigments. By contrast, several groups, such as the diatoms, dinoflagellates, yellow-green algae and higher plants, do not contain a single xanthophyll in common (Strain and Manning, 1943b).

Because of the abundance and wide distribution of diatoms and dinoflagellates, the principal xanthophylls of these organisms, fucoxanthin and peridinin, must comprise a major portion of the total amount of xanthophylls occurring in the world's vegetation. In the dinoflagellate, the proportion of peridinin relative to the two chlorophylls was quite large. On account of the strong absorption of light by peridinin in the spectral region where absorption by the chlorophylls is weak, studies of the photosynthetic activity of dinoflagellates in light of various wavelengths might yield information regarding the role of this unique xanthophyll in photosynthesis.

Interpretation of the photochemical activity of the individual leaf pigments depends upon knowledge of the physical state and spectral properties of the several pigments in the leaf itself. Spectral properties of the pigments determined in any one solvent or in colloidal suspension may not yield results precisely applicable to calculation of the absorption by each pigment in the living organism.

The variation in pigments between members of different algal classes, together with the constancy of the pigments in different members of a single class, provides a promising approach to the problem of phylogenetic relationship between the various classes of algae. For example, brown algae contain at least one xanthophyll, violaxanthin, common to the higher green plants, whereas diatoms and dinoflagellates contain none. Tribonema, sometimes considered closely related to the diatoms, contains none of the principal diatom xanthophylls, nor chlorophyll *c*. None of the algae investigated contain lutein, the common xanthophyll of green algae and of higher plants. These and other phylogenetic aspects of our observations on the chloroplast pigments of plants will be discussed elsewhere.

Adsorption phenomena. Reversal of the relative positions of adsorbed substances with changes in solvents and adsorbents indicates disproportionate variations in the adsorbability. It follows that the relative adsorbability of chemical substances is not determined by chemical structure alone; hence, great care must be exercised if deductions of molecular structure are to be based upon relative adsorbability (Strain, 1942a; 1942b).

Variations of the relative adsorbabilities of chemical compounds with changes in adsorbents and solvents suggest precautions to be employed in the use of the chromatographic adsorption method. For instance, if one solvent causes a given

pair of substances to be adsorbed in one sequence and if another solvent causes the same substances to be adsorbed in the inverse order, then there should be at least one mixture of the two solvents that will not effect a separation of the pair of adsorbed substances. Tests of the selectivity of a given adsorbent for separation of a mixture of given compounds can not, therefore, be regarded as conclusive unless different solvents and various mixtures of these solvents are used to develop the chromatograms. Determination of the homogeneity of chemical substances and the comparison of substances suspected of being identical through use of chromatographic adsorption methods (Strain, 1942b) will be most effective when various adsorbents and solvents are employed. Reversal of relative adsorbability with changes in the solvents suggests that the selectivity of the adsorbent may be just as dependent upon the nature of the solvents employed as upon the inherent properties of the adsorbent itself. Unless special precautions are taken, these changes in relative adsorbability increase the chances for confusion and error when substances are identified by the so-called mixed chromatogram (Strain, 1942b).

Most of the plant pigments that have been separated upon adsorption columns have yielded bands or zones with the highest concentration of pigment in the leading portions and with diminishing concentrations in the trailing portions (Strain, 1942a). The inverse behavior of adsorbed diadinoxanthin is difficult to interpret in relation to the theories regarding the distribution of an adsorbed pigment upon the columns (Strain, 1942a; DeVault, 1943).

The great color change observed upon adsorption of fucoxanthin and peridinin, in contrast to the slight change observed upon adsorption of neoxanthin, may result from interaction between the adsorbent and the pigments rather than from increased concentration of the xanthophylls at the interface. This effect may be analogous to that produced by addition of polar solvents to solutions of peridinin in nonpolar solvents (Figs. 3 and 6). It emphasizes the desirability of additional knowledge concerning the state of the pigments within the leaf, especially for interpretation of the photosynthetic reactions in various spectral regions (Dutton and Manning, 1941) and for interpretation of color changes that occur when algae are killed. The strong adsorption of some of the xanthophylls at the interface between water and petroleum ether may be utilized for separation of these pigments from other less adsorped constituents in the extracts of plants (Strain, 1943).

Summary

Carotenoid pigments of several groups of algae have been obtained through utilization of the chromatographic adsorption method. The selectivity of this method and the relative positions of the pigments in the columns have been found to vary with the solvents and adsorbents that were employed.

Xanthophylls of algae represent a large proportion of the carotenoid pigments produced in the world's vegetation. Most of the algal xanthophylls were readily convertible, reversibly, into one or more isomers that were separated on the adsorption columns. The principal xanthophylls were the more stable, presumably *trans*, isomers. Some of the labile isomers also appeared to be normal constituents of the cells. All the algal xanthophylls were unesterified.

The following xanthophylls have been obtained from each of six species of diatoms: diatoxanthin, diadinoxanthin (both new xanthophylls), fucoxanthin, neo-

fucoxanthin A, and neofucoxanthin B. From some eight species of brown algae there were obtained: diatoxanthin (occasionally, in traces), diadinoxanthin (occasionally, in traces), violaxanthin, a flavoxanthin-like xanthophyll, fucoxanthin, neofucoxanthin A, neofucoxanthin B and traces of other xanthophylls. The dinoflagellate *Peridinium cinctum* and an alga inhabiting a sea-anemone yielded the following xanthophylls: diadinoxanthin, dinoxanthin (a new xanthophyll), neodiadinoxanthin (a new xanthophyll), neodinoxanthin (a new xanthophyll), peridinin, and two or three isomers of peridinin. A yellow-green alga, *Tribonema bombycinum*, did not contain fucoxanthin, peridinin or chlorophyll *c*.

None of the algae examined contained lutein. All the algae examined contained β-carotene as the principal polyene hydrocarbon. In only one species, the diatom *Navicula torquatum*, were appreciable quantities of another carotene ϵ-carotene, observed.

A new xanthophyll, tareoxanthin, was obtained from flowers of the dandelion. A flavoxanthin-like xanthophyll from these flowers may not be identical with flavoxanthin from other sources. Violeoxanthin, a new xanthophyll, was isolated from the flowers of the pansy. Improved methods for the isolation of neoxanthin and violaxanthin from leaves are described. The violaxanthin b of leaves and violaxanthin of pansies are chromatographically identical.

Several groups of xanthophylls with almost identical characteristic spectral absorption curves have now been found. Neoxanthin, tareoxanthin and violeoxanthin fall into one group; violaxanthin, taraxanthin, and dinoxanthin comprise another group; and there are indications of several flavoxanthin-like pigments.

The solvent has a pronounced effect on the shape of the spectral absorption curves of peridinin and of fucoxanthin. In addition to indicating an effect of the solvent on the structure of the pigment molecule, this phenomenon also emphasizes the importance of knowledge concerning the spectral properties of the xanthophylls in the living plant, especially if these spectral characteristics are to be employed in an analysis of the photochemical activity of the plant pigments.

Of the pigments comprising the photosynthetic apparatus, the xanthophylls are subject to the greatest variation. Nearly two dozen of these pigments have been found in the green parts of various plants.

All members of the Division of Plant Biology contributed invaluable discussion of the methods and results herein described. Dr. Gilbert M. Smith of Stanford University and Dr. H. W. Graham of Mills College aided in the identification of the algae.

LITERATURE CITED

CARTER, P. W., I. M. HEILBRON, AND B. LYTHGOE, 1939. The lipochromes and sterols of the algal classes. *Proc. Roy. Soc., Series B*, **128**: 82–109.

DEVAULT, D., 1943. The theory of chromatography. *Jour. Amer. Chem. Soc.*, **65**: 532–540.

DUTTON, H. J., AND W. M. MANNING, 1941. Evidence for carotenoid-sensitized photosynthesis in the diatom Nitzschia closterium. *Amer. Jour. Bot.*, **28**: 516–526.

FULTON, J. F., JR., 1922. Animal chlorophyll: its relation to haemoglobin and to other animal pigments. *Quart. Jour. Micr. Sci.*, **66**: 339–396.

HANDKE, H. H., 1941. Hydrographische und biochemische Untersuchungen über die Plankton-Produktionskraft des süssen Sees bei Halle. *Bot. Arch.*, **42**: 149–200.

HEILBRON, I. M., 1942. Some aspects of algal chemistry. *Nature*, **149**: 398–400; *Jour. Chem. Soc.*, 79–89.

HEILBRON, I. M., H. JACKSON, AND R. N. JONES, 1935. The lipochromes of sea anemones. I. Carotenoid pigments of Actinia equina, Anemonia sulcata, Actinoloba dianthus and Tealia felina. *Biochem. Jour.*, **29**: 1384–1397.

HEILBRON, I. M., AND B. LYTHGOE, 1936. The chemistry of the algae. The carotenoid pigments of Oscillatoria rubrescens. *Jour. Chem. Soc.*, 1376–1380.

HEILBRON, I. M., E. G. PARRY, AND R. F. PHIPERS, 1935. The relationship between certain algal constituents. *Biochem. Jour.*, **29**: 1376–1381.

HEILBRON, I. M., AND R. F. PHIPERS, 1935. The lipochromes of Fucus vesiculosus. *Biochem. Jour.*, **29**: 1369–1375.

KARRER, P., AND J. RUTSCHMANN, 1942a. Über die Phytoxanthine der Löwenzahnblüten, Flavoxanthin. *Helv. chim. Acta*, **25**: 1144–1149.

KARRER, P., AND J. RUTSCHMANN, 1942b. Auroxanthin, ein kurzwellig absorbierender Carotinfarbstoff. *Helv. chim. Acta*, **25**: 1624–1627.

KUHN, R., AND H. BROCKMANN, 1932. Flavoxanthin. *Zeit. physiol. Chem.*, **213**: 192–198.

KUHN, R., AND E. LEDERER, 1931. Taraxanthin, ein neues Xanthophyll mit 4 Sauerstoffatomen. *Zeit. physiol. Chem.*, **200**: 108–114.

KUHN, R., AND A. WINTERSTEIN, 1931. Viola-xanthin, das Xanthophyll des gelben Stiefmütterchens (Viola tricolor). *Ber. chem. Ges.*, **64**: 326–332.

KYLIN, H., 1927. Über die karotinoiden Farbstoffe der Algen. *Zeit. physiol. Chem.*, **166**: 39–77.

KYLIN, H., 1939. Bemerkungen über die carotinoiden Farbstoffe der Algen. *Kgl. Fysiograf. Sällskap. Lund, Förh.*, **9**: 213–231.

LEROSEN, A. L., 1942. A method for standardization of chromatographic analysis. *Jour. Amer. Chem. Soc.*, **64**: 1905–1907.

MONTFORT, C., 1940. Die Photosynthese brauner Zellen im Zusammenwirken von Chlorophyll und Carotinoiden. *Zeit. physik. Chem., A* **186**: 57–93.

PACE, N., 1941. Pigments of the marine diatom Nitzschia closterium. *Jour. Biol. Chem.*, **140**: 483–489.

PALMER, L. S., 1922. *Carotinoids and related pigments.* Chemical Catalog Co., Inc., New York.

POLGÁR, A., AND L. ZECHMEISTER, 1942. Isomerization of β-carotene. Isolation of an isomer with increased adsorption affinity. *Jour. Amer. Chem. Soc.*, **64**: 1856–1861.

SCHÜTT, F., 1890. Ueber Peridineenfarbstoffe. *Ber. bot. Ges.*, **8**: 9–32.

SEYBOLD, A., AND K. EGLE, 1938. Quantitative Untersuchungen über Chlorophyll und Carotinoide der Meeresalgen. *Jahrb. wissensch. Bot.*, **86**: 50–80.

SEYBOLD, A., K. EGLE, AND W. HÜLSBRUCH, 1941. Chlorophyll- und Carotinoidbestimmungen von Süsswasseralgen. *Bot. Arch.*, **42**: 239–253.

SMITH, J. H. C., 1936. A comparison of absorption spectra measurements on α-carotene, β-carotene and lycopene. *Jour. Amer. Chem. Soc.*, **58**: 247–255.

STRAIN, H. H., 1938a. Leaf xanthophylls. *Carnegie Inst. of Washington, Publ. No. 490.*

STRAIN, H. H., 1938b. Eschscholtzxanthin: a new xanthophyll from the petals of the California poppy, Eschscholtzia californica. *Jour. Biol. Chem.*, **123**: 425–437.

STRAIN, H. H., 1941. Isomerization of polyene acids and carotenoids. Preparation of β-eleostearic and β-licanic acids. *Jour. Amer. Chem. Soc.*, **63**: 3448–3452.

STRAIN, H. H., 1942a. Problems in chromatography and in colloid chemistry illustrated by leaf pigments. *Jour. Phys. Chem.*, **46**: 1151–1161.

STRAIN, H. H., 1942b. *Chromatographic adsorption analysis.* Interscience Publishers, Inc., New York.

STRAIN, H. H., 1943. Improved methods of pigment analysis. *Carnegie Inst. of Washington, Year Book*, **42**: 89–90.

STRAIN, H. H., AND W. M. MANNING, 1942a. Chlorofucine (chlorophyll γ), a green pigment of diatoms and brown algae. *Jour. Biol. Chem.*, **144**: 625–636.

STRAIN, H. H., AND W. M. MANNING, 1942b. The occurrence and interconversion of various fucoxanthins. *Jour. Amer. Chem. Soc.*, **64**: 1235.

STRAIN, H. H., AND W. M. MANNING, 1943a. A unique polyene pigment of the marine diatom Nitzschia closterium. *Jour. Amer. Chem. Soc.*, **65**: 2258–2259.

STRAIN, H. H., AND W. M. MANNING, 1943b. Pigments of algae. *Carnegie Inst. of Washington, Year Book*, **42**: 79–83.

STRAIN, H. H., W. M. MANNING, AND G. HARDIN, 1943. Chlorophyll *c* (chlorofucine) of diatoms and dinoflagellates. *Jour. Biol. Chem.*, **148**: 655–668.

TSWETT, M., 1906a. Zur Kenntnis der Phaeophyceenfarbstoffe. *Ber. bot. Ges.*, **24**: 235–244.

TSWETT, M., 1906b. Adsorptionsanalyse und chromatographische Methode. Anwendung auf die Chemie des Chlorophylls. *Ber. bot. Ges.*, **24**: 384–393.

WALKER, O., 1935. I. Überblick über die Chemie der Carotinoide und ihre verbreitung in der Natur. II. Untersuchungen über carotinoide. Inaugural-dissertation. Zürich.

WILLSTÄTTER, R., AND H. J. PAGE, 1914. Über die Pigmente der Braunalgen. *Ann. Chem.*, **404**: 237–271.

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